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Remarks

Applicants appreciate the Examiner's withdrawal of the rejection of claims 36-41, 66, 88, and 92 under 35 U.S.C. § 102(a) and the rejections of claims 36, 38-41, 66, 88, and 92 under 35 U.S.C. § 103(a).

The Amendments to the Claims

Independent claims 66, 88, and 92 as amended recite that the live *P. haemolytica* bacterium "when in a physiological environment" expresses the recited mutant leukotoxin molecule. This amendment is supported *inter alia* on page 13, lines 4-6: "A new protein of approximately 65 kDa was detected in the culture supernatant of this mutant by SDS-PAGE, consistent with the predicted molecular weight of the deleted product."

New dependent claims 96-100 recite that the live bacterium is lyophilized (claims 96, 98, and 100) or reconstituted from a lyophilized preparation (claims 97, 99, and 101). These dependent claims are supported on page 4, lines 14-15: "The bacteria in the vaccine formulation can be live, lyophilized, lyophilized and reconstituted, or killed."

Independent claims 81, 91, and 95 have been amended to recite "a vaccine formulation" that comprises "at least two sources of a form of a leukotoxin molecule, wherein a first source is a killed *P. haemolytica* bacterium, wherein a live form of the killed bacterium (a) expresses no biologically active leukotoxin, (b) expresses a form of leukotoxin molecule which is a deletion mutant of about 66 kDa which lacks amino acids 34 to 378 and which induces antibodies which specifically bind to and neutralize biologically active leukotoxin, and (c) contains no non-*P. haemolytica* DNA, and wherein a second source comprises the leukotoxin molecule expressed by the live form of the killed bacterium." New dependent claims 102-104 recite that the second

source of the form of a leukotoxin molecule is selected from the group consisting of purified protein, a bacterial lysate, a bacterial extract, and a culture supernatant.

The amendment and the new claims are supported on page 4, lines 15-17: "Moreover, bacterial lysates, extracts or culture supernatants which contain the LtkA deletion protein can be used in the vaccine formulation. Purified protein can also be used, if desired."

Applicants have more claims (claims 67-80, 89, 90, 93, and 94) than those added by this amendment. The new claims and amendments were not presented previously because Applicants believed the arguments in the last response were sufficient to overcome the rejection. The amendments do not recite new subject matter and do not require a new search. The new dependent claims recite subject matter previously recited in canceled independent claims. The new dependent claims clarify the claimed subject matter by indicating that "lyophilized" bacteria and bacteria "reconstituted from a lyophilized preparation" are species of "live" bacteria. The amendments also present the claims in better form for appeal.

Applicants respectfully request entry of the amendments and the new dependent claims.

The Obviousness-Type Double Patenting Rejections of Claims 36-41 and 66-95

Claims 36-41 and 66-95 stand rejected under the judicially created doctrine of obviousness-type double patenting over claims 1-9 of U.S. Patent 6,495,145 and claims 22-29 of co-pending application Serial No. 09/736,169.

Claims 67-80, 89, 90, 93, and 94 have been canceled. To expedite prosecution of the remaining claims, a Terminal Disclaimer under 37 C.F.R. § 1.321 over claims 1-9 of U.S. Patent 6,495,145 accompanies this response. Serial No. 09/736,169 is abandoned; a non-final rejection

to which Applicants did not respond was mailed in that application on December 4, 2002. Thus, no terminal disclaimer over Serial No. 09/736,169 is needed.

Applicants respectfully request withdrawal of the rejection.

The Rejection of Claims 66-95 Under 35 U.S.C. § 112, second paragraph

Claims 66-95 stand rejected under 35 U.S.C. § 112, second paragraph, as indefinite. Claims 67-80, 89, 90, 93, and 94 have been canceled. Applicants respectfully traverse the rejection of claims 66, 81-88, 91, 92, and 95.

The second paragraph of 35 U.S.C. § 112 states that:

> The specification shall conclude with one or more claims particularly pointing out and distinctly claiming the subject matter which the applicant regards as his invention.

It is well settled that a claim must "reasonably apprise those skilled in the art both of the utilization and scope of the invention." *Georgia-Pacific Corp. v. United States Plywood Corp.*, 258 F.2d 124, 134-38, 118 U.S.P.Q. 122, 130 (2d Cir. 1958), *cert. denied*, 358 U.S. 884 (1958). Claims 66, 81-88, 91, 92, and 95 meet this standard.

The Final Office Action asserts that claims 81-87, 91, and 95 are be vague and indefinite because claim 81 recites that a killed bacterium is administered to the ruminant. The Final Office Action requests clarification of how a vaccine preparation comprising the recited killed bacterium differs from a vaccine preparation comprising a killed wild-type *P. haemolytica*. Independent claims 81, 91, and 95 have been amended to recite a vaccine formulation that contains the mutant leukotoxin protein as well as the killed bacterium. Thus, at least one difference between the claimed vaccine formulation and a vaccine formulation comprising a

killed wild-type *P. haemolytica* is that the claimed vaccine formulation contains the mutant leukotoxin protein.

The Final Office Action asserts that claims 67-80, 90, 93, and 94 are vague and indefinite because "it is unclear what is meant by administering a 'lyophilized' . . . versus a 'lyophilized and reconstituted' . . . *P. haemolytica* vaccine." Final Office Action at page 3, first full paragraph. Claims 67-80, 90, 93, and 94 have been canceled. Similar subject matter, however, is recited in new dependent claims 96-101. New dependent claims 96, 98, and 100 recite that the live bacterium of claim 66 is lyophilized. New dependent claims 97, 99, and 101 recite that the live bacterium of claim 66 is "reconstituted from a lyophilized preparation."

The term "lyophilized" is well known in the art and means "freeze-dried." Lyophilization is a common method of preserving a live bacterial vaccine until it is administered. *See, e.g.*, the abstract of Confer *et al.*, *Am. J. Vet. Res. 47*, 1853-57, 1986 (Attachment 1). Those skilled in the art understand that a live bacterium reconstituted from a lyophilized preparation means that the recited live bacterium was lyophilized but has now been placed in a suitable liquid medium in which the live bacterium can function. *Id.*: "Lyophilized P. haemolytica was reconstituted and used as a live vaccine in 3 experiments."

The Final Office Action requests clarification of how a lyophilized bacterium "would not degrade while sitting on animal feed." The Final Office Action also states it is unclear "how a dry powder of a lyophilized bacterium would allow for the active ingredient, the modified leukotoxin, to be expressed in the ruminant." Office Action at page 3, first full paragraph.

First, whether some amount of dry lyophilized bacteria "degrade while sitting on animal feed" is not relevant to whether or not the claims are definite because the claims recite no time period during which the recited bacteria must not degrade. Second, bacteria in lyophilized

preparations are still "live." Lyophilized live bacteria administered dry, for example as top-dressing on feed, become reconstituted in the animal. When the dry, lyophilized bacteria come in contact with a moist environment in a ruminant (*e.g.*, an oral, pharyngeal, or nasal surface), it will become wet and reconstituted. The reconstituted bacteria can multiply on the host mucosa and can express the mutant leukotoxin. See paragraphs 22 and 23 of the accompanying declaration of Dr. Briggs, which describes a field trial in which lyophilized bacteria were top-dressed on feed. Administration of dry lyophilized live bacteria in this manner "dramatically reduced nasal colonization by virulent *M. haemolytica* serotype 1 ($p < 0.001$)." Paragraph 23 of the declaration. Thus, dry lyophilized live bacteria do allow for the active ingredient, the modified leukotoxin, to be expressed in the ruminant.

The Final Office Action asserts that claims 74, 90, and 94 are vague and indefinite because these claims recite that the bacterium is reconstituted prior to administration. Claims 74, 90, and 94 have been canceled; however, similar subject matter is recited in new dependent claims 97, 99, and 101. As explained above, "reconstituted" simply means that the lyophilized bacteria are placed into a suitable liquid before being administered to a ruminant. As the Final Office Action notes, the claims encompass reconstitution in adjuvant as well as reconstitution in growth medium.

The Final Office Action also questions how administration of a lyophilized and reconstituted bacterium differs from administration of a lyophilized bacterium. A lyophilized bacterium is a dry, freeze-dried bacterium; a reconstituted bacterium is wet (*i.e.*, is in a liquid). *See* Confer *et al.* (Attachment 1).

Claims 66, 81-88, 91, 92, 95, and new dependent claims 96-101 are clear and definite because they reasonably convey to one skilled in the art what the invention is. Applicants respectfully request withdrawal of the rejection.

The Rejection of Claims 67-87, 89-91, and 93-95 Under 35 U.S.C. § 112, first paragraph

Claims 67-87, 89-91, and 93-95 stand rejected under 35 U.S.C. § 112, first paragraph, as not enabled. Claims 67-80, 89, 90, 93, and 94 have been canceled. Applicants respectfully traverse the rejection of claims 81-87, 91, and 95.

The Final Office Action acknowledges that claims to methods of inducing immunity using live forms of the bacterium recited in claim 66, as well as claims to vaccines and feeds comprising the bacterium, are enabled. Yet the Office Action questions the enablement of similar claims (now canceled but replaced with claims with the same recitations) that recite lyophilized, lyophilized and reconstituted, or killed bacteria.

Use of lyophilized bacteria or lyophilized and reconstituted bacteria

The pending claims have been amended to clarify that lyophilized bacteria and bacteria reconstituted from lyophilized preparations are species of live bacteria. The accompanying declaration of Dr. Robert Briggs describes four field trials in which both types of vaccines (containing dry, lyophilized bacteria and containing bacteria reconstituted from a lyophilized preparation) were administered to ruminants and proved effective in inducing immunity to pneumonic pasteurellosis. In each trial, the bacterial strain in the vaccines was D153ΔlktA34-378; these are *P. haemolytica* that do not express a biologically active leukotoxin, express a mutant leukotoxin protein that lacks amino acids 34-378, and contain no non-*P. haemolytica* DNA. The collective results of the four trials demonstrates that such bacteria, whether

administered dry or reconstituted, induce immunity to pneumonic pasteurellosis as measured by reduced mortality, increased serum antibody titers against *P. haemolytica*, or reduced nasal colonization by *P. haemolytica*.[1]

Paragraphs 4-7 and 11-21 of the declaration describe two field trials in which bacteria reconstituted from a lyophilized preparation were top-dressed on feed. In one trial, the calves to which the bacteria were administered gained weight and had reduced mortality (4% of the population vs 16% of the unvaccinated population) from *M. haemolytica* infection. See paragraphs 4-7 of the declaration. In another trial, the calves to which the bacteria were administered had increased serum antibody titers against *M. haemolytica* and increased weight gain when compared with unvaccinated animals. See paragraphs 11-21 of the declaration.

Paragraphs 8-10 of the declaration describe a field trial in which bacteria reconstituted from a lyophilized preparation were administered intranasally. The calves to which the bacteria were administered had increased serum antibody titers against *M. haemolytica* and increased weight gain when compared with unvaccinated calves.

Paragraphs 22 and 23 of the declaration describe a field trial in which lyophilized bacteria were top-dressed on feed and administered to calves. Administration of dry lyophilized live bacteria in this manner "dramatically reduced nasal colonization by virulent *M. haemolytica* serotype 1 ($p < 0.001$)." Paragraph 23 of the declaration.

The results of the trials described in Dr. Briggs' declaration demonstrate that vaccines containing lyophilized live bacteria and reconstituted, previously lyophilized live bacteria work as described in the specification.

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[1] Since this application was filed, *P. haemolytica* has been renamed "*Mannheimia haemolytica*." The declaration uses the new terminology.

Use of vaccine formulations containing killed bacteria

Independent claims 81, 91, and 95 as amended to recite a vaccine formulation that comprises at least two sources of a form of a leukotoxin molecule. The first source is a killed *P. haemolytica* bacterium, wherein a live form of the killed bacterium (a) expresses no biologically active leukotoxin, (b) expresses a form of leukotoxin molecule which is a deletion mutant of about 66 kDa which lacks amino acids 34 to 378 and which induces antibodies which specifically bind to and neutralize biologically active leukotoxin, and (c) contains no non-*P. haemolytica* DNA. The second source comprises the leukotoxin molecule expressed by the live form of the killed bacterium. The claimed vaccine preparation contains an active agent (the leukotoxin deletion mutant protein) that the specification teaches is useful as a vaccine. Specification at page 3, line 15 to page 4, line 31.

Vaccine preparations containing a killed bacterium (bacterin) and an inactivated protein toxin (toxoid) for inducing immunity against *P. haemolytica* were well known in the art at the priority date of this application (September 25, 1997). See Srinand *et al.*, *Vet. Microbiol. 49*, 181-95, 1996 (Attachment 2); and Confer, *Vet. Microbiol. 37*, 353-68, 1993 (Attachment 3). Thus, those of skill in the art at that time knew how to make and use such preparations to induce immunity against *P. haemolytica*. In fact, such bacterin-toxoid preparations are still commercially available (*e.g.*, "One Shot," referred to in Srinand *et al.*, above).

The specification, together with the skill in the art at the priority date of this application, enables making and using vaccines containing the recited live, lyophilized live, and reconstituted lyophilized live bacteria, as well as vaccines containing the recited killed bacteria and leukotoxin mutant protein. Applicants respectfully request withdrawal of the rejection.

Respectfully submitted,
BANNER & WITCOFF, LTD.

Date: March 22, 2004

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Immunologic response and resistance to experimentally induced pneumonic pasteurellosis in cattle vaccinated with various dosages of lyophilized *Pasteurella haemolytica*

A. W. Confer, DVM, PhD; R. J. Panciera, DVM, PhD; M. J. Gentry, PhD; R. W. Fulton, DVM, PhD

SUMMARY

Pasteurella haemolytica was lyophilized in an enriched soybean polypeptone broth. Lyophilization in this medium resulted in a mean 10-fold loss in *P haemolytica* viability, as opposed to up to a 10^4-fold loss in viability when other media were used. Lyophilized *P haemolytica* was reconstituted and used as a live vaccine in 3 experiments. Calves were challenge exposed by transthoracic injection with virulent *P haemolytica*. In experiment 1, 2 subcutaneous injections (7-day interval between injections) with 5 ml of recently harvested (1×10^9 colony-forming units [CFU]/ml) or lyophilized (1×10^8 CFU/ml) *P haemolytica* significantly ($P < 0.001$) enhanced resistance against challenge exposure, compared with resistance in calves given saline solution or sterile medium (control calves) or calves vaccinated with lyophilized organisms at a concentration of 1×10^6 CFU/ml. In experiment two, 1, 2, or 5 ml of lyophilized *P haemolytica* (1×10^8 CFU/ml) significantly ($P < 0.05$) enhanced resistance, compared with resistance in calves given saline solution (control calves). In experiment three, 1 or 2 injections of lyophilized *P haemolytica* significantly ($P < 0.01$) enhanced resistance against challenge exposure, compared with that of calves given saline solution. The mean lesion score for calves given 1 injection was not significantly higher than the mean lesion score for the group given 2 injections. Vaccination with lyophilized *P haemolytica* vaccine caused significant ($P < 0.05$) increases in serum antibody to *P haemolytica* somatic antigens, to a carbohydrate-protein subunit of the organism, and to leukotoxin.

Bovine pneumonic pasteurellosis (shipping fever) is a severe fibrinous pneumonia of feedlot cattle.[1] *Pasteurella haemolytica* biotype A serotype 1 and, to a lesser extent, *P multocida* type 3 are important in the development of the disease.[2]

Live *P haemolytica* vaccines can protect calves against experimentally induced and possibly naturally acquired pneumonic pasteurellosis.[3-10] Such vaccines contain organisms that have been lyophilized or recently harvested from bacteriologic cultures. Recently harvested *P haemolytica* has been administered via aerosol, subcutaneous, or intradermal routes.[4,5,9] Lyophilized *P haemolytica* vaccines consist of chemically altered, streptomycin-dependent, or modified-live organisms and have been given intradermally or IM.[3,6-8,10]

The antibody response of calves to *P haemolytica* somatic antigens or to a carbohydrate-protein subunit (CPS) antigen of the organism (separated from a saline solution extract) and the leukotoxin neutralization (LN) response of calves after vaccination with recently harvested *P haemolytica* cultures have been characterized, and high antibody responses to CPS or leukotoxin have correlated with resistance to challenge exposure.[4,9,11,12]

Purposes of the present study were to evaluate the dosage-related reactions to a lyophilized *P haemolytica* vaccine, both systematically and at the injection site, resistance to intrapulmonic challenge exposure, and antibody response in calves, and to determine the effects of various lyophilization procedures on the viability of *P haemolytica*.

Materials and Methods

Calves—Sixty-four weaned beef calves, 7 to 9 months old, were obtained from a closed herd and transported to holding pens. Husbandry of the calves was as previously reported.[13]

Pasteurella haemolytica—*Pasteurella haemolytica* biotype A serotype 1 was isolated originally from the trachea of a feedlot calf and was grown on supplemented brain-heart infusion (BHI) agar for 24 hours at 37 C in a 5% CO_2 environment, as previously described.[14] Cultures were harvested in phosphate-buffered saline solution (PBSS; 0.01*M*, pH 7.4) at an approximate concentration of 1×10^9 colony-forming units (CFU)/ml, as determined photometrically. Actual CFU per milliliter were determined for each culture, using a spot plate-counting technique.[14]

Lyophilized vaccine—Lyophilization medium was prepared by adding 14.8 g of BHI,* 2 g of casamino acids,* and 2 g of NaCl

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to 280 ml of distilled H_2O. The medium was heated to 50 C and 20 ml of a 20% yeast hydrolysate solution,[b] 40 ml of fetal bovine serum (previously heat inactivated at 56 C for 30 min), 20 ml of a 10% bovine hemin[c] solution, 40 ml of 10% soybean polypeptone solution[d] and 80 g of dextrose was added to the medium, then the medium was autoclaved.[15] Henceforth, the medium will be referred to as polypeptone lyophilization medium (PLM).

The lyophilized vaccine was made by suspending *P haemolytica* (harvested after 24 hours growth on BHI infusion agar) in PLM (volume: volume = 1) at a concentration of approximately 0.9 to 3.0 × 10^9 CFU/ml. The suspension was shell frozen over cold alcohol and lyophilized. After lyophilization and storage at −20 C, several vials were reconstituted with distilled H_2O. The number of viable *P haemolytica* were determined periodically for 24 hours, using reconstituted lyophilized cultures kept at 4 C or 25 C. The viability of cultures before and after lyophilization were compared with cultures prepared in sterile skim milk, bovine serum albumin (BSA; 5%, 10%, 20%, and 35%) in PBSS, dextrose (10% and 20%) in PBSS, and combinations of BSA (10% and 20%) and dextrose (10% and 20%) in PBSS.[15,17]

Experimental design—Three experiments (1, 2, and 3) were conducted. All vaccinations were given subcutaneously in the caudal cervical region. In each experiment, 2 to 4 calves were vaccinated (day 0) with 5 ml of a suspension of live *P haemolytica* that had been recently harvested from BHI agar plates within 1 to 2 hours before injection.[9] The experiments used 2 different lots of lyophilized vaccine that had been stored for 1 to 5 months before use. After initial vaccination (day 21), calves were transthoracically inoculated (challenge exposed) with 5 ml of a suspension of *P haemolytica* (concentration was from 0.9 to 4.5 × 10^9 CFU/ml) into each caudal lung lobe, as previously described.[18] Four days after inoculation, all calves were slaughtered and lung lesions were evaluated by use of a lesion-scoring technique.[9] Scores of 0 to 20 were based on size and spread of lesions, with higher scores indicating the more severe lesions.

Rectal temperatures were determined on days 0 through 10 and days 21 through 24 of each experiment. Vaccination sites were examined on days 0 through 10 and on day 21. Blood samples were collected on days 0 and 21.

Experiment 1—To compare the protection provided calves by 2 different concentrations of lyophilized *P haemolytica* cultures given on days 0 and 7, 4 calves were vaccinated with PBSS (group 1), 4 calves were vaccinated with sterile PLM (group 2), and 4 calves were vaccinated with a recently harvested *P haemolytica* culture (1 × 10^9 CFU/ml; group 3). Calves in groups 4 and 5 (6 calves/group) were vaccinated with reconstituted lyophilized *P haemolytica* at concentrations of 1 × 10^9 CFU/ml (group 4) or 1 × 10^8 CFU/ml (group 5). On day 10, group 1 and group 2 calves (control calves) were skin tested for a delayed-type hypersensitivity to the soybean polypeptone solution; 1 ng to 100 ng of the polypeptone was injected intradermally. Each calf was challenge exposed with *P haemolytica* at 0.9 × 10^9 CFU/ml. Serum samples collected from group 2 calves on days 0 and 21, were evaluated for antibody against the polypeptone and yeast hydrolysate by use of an enzyme-linked immunosorbent assay (ELISA), similar to that described previously.[11] Plates were coated with polypeptone or yeast hydrolysate at a concentration of 100 μg/ml (dry weight). Serum samples were diluted 1:100 in the assay.

Experiment 2—To compare 3 different volumes of a single concentration of lyophilized vaccine given on days 0 and 7, 5 calves were vaccinated with PBSS (group 6) and 3 calves were vaccinated with 5 ml of a recently harvested *P haemolytica* culture (1 × 10^9 CFU/ml; group 7). Fifteen calves were allotted among 3 groups (5 calves/group) and vaccinated with 5 ml (group 8), 2 ml (group 9), or 1 ml (group 10) of lyophilized vaccine (1 × 10^8 CFU/ml). Each calf was challenge exposed with *P haemolytica* at a concentration of 4.5 × 10^9 CFU/ml.

Experiment 3—To compare the efficacy of 1 or 2 injections of 2 ml of lyophilized vaccine (1 × 10^8 CFU/ml), 5 calves were vaccinated with PBSS (group 11) and 2 calves were vaccinated with recently harvested *P haemolytica* (1 × 10^9 CFU/ml; group 12). Five calves (group 13) were vaccinated on days 0 and 7 and 5 calves (group 14) were vaccinated on day 0 with lyophilized vaccine. Each calf was challenge exposed with *P haemolytica* at a concentration of 1.0 × 10^9 CFU/ml.

Serologic evaluation—Serum samples were evaluated for antibodies against *P haemolytica* somatic antigens, using a quantitative fluorometric immunoassay (FIAX).[19,*] Antigen for the FIAX was a formalin-killed *P haemolytica* serotype 1 from a 22-hour culture. Titer equivalents were calculated for each sample by comparison with a standard curve constructed by use of sera of known end-point titers.

Serum LN titers against *P haemolytica* were determined by use of a visual microtiter neutralization assay.[20] Titers were expressed as the reciprocal of the last serum dilution that neutralized leukotoxin.

The serum antibody response to a high molecular weight (> 200 kilodaltons) CPS separated from a saline solution extract of *P haemolytica* was determined by use of an ELISA.[11] Antibody responses were expressed as the absorbance at 490 nm (A_{490}) for unknown sera (1:250 dilution in PBSS with 1% BSA) minus the A_{490} for PBSS-BSA controls.

Statistical analyses—Differences in mean antibody responses and lesion scores were analyzed by use of a Student's *t* test.[21] Antibody titers were compared with lesion scores by use of linear regression analysis.

Results

Lyophilization—Lyophilization of *P haemolytica* in PLM resulted in a mean 10-fold decrease in CFU/ml. Cultures lyophilized in skim milk had a similar decrease in CFU per milliliter, whereas cultures lyophilized in PBSS containing BSA (5% to 35%), dextrose (5 to 20%), or BSA-dextrose combinations had a mean 10^4-fold decrease in CFU/ml.

Viability counts for *P haemolytica* from reconstituted PLM kept at 4 C were similar at the time of reconstitution (mean = 2.8 × 10^9 CFU/ml) and 24 hours after reconstitution (mean = 3.6 × 10^8 CFU/ml). Maintenance of reconstituted vaccines at room temperature (25 C) did not result in loss in viability between the time of reconstitution (mean = 1.3 × 10^8 CFU/ml) and 8 hours after reconstitution (mean = 1.3 × 10^8 CFU/ml). However, frequent sampling from vials resulted in bacterial contamination, such that viability counts could not be determined on the 24-hour samples.

Clinical signs after vaccination—Vaccination with recently harvested or lyophilized *P haemolytica* often resulted in soft swellings, 4 to 8 cm in diameter, at the first injection site. Local reactions usually were not detectable at the site of the 2nd injection. Rectal temperatures increased 1 to 2.5 C for up to 72 hours after the 1st vaccination, but usually remained normal after the 2nd vaccination. In experiment one, 2 calves had a transient

[b] ICN Biomedicals Inc, Cleveland, Ohio.
[c] Sigma Chemical Co, St Louis, Mo.
[d] Daigo Brand, Japan.
[*] International Diagnostic Technology Inc, Santa Clara, Calif.

thoracic limb lameness, with mild subcutaneous edema after the 1st vaccination with the lyophilized vaccine (1×10^6 CFU/ml). A delayed-type hypersensitivity response to the soybean polypeptone was not detected. The mean (± SD) antibody response to polypeptone was 0.06 ± 0.01 on day 0 and was 0.07 ± 0.01 on day 21. The mean antibody response to yeast hydrolysate was 0.09 ± 0.02 on day 0 and was 0.11 ± 0.03 on day 21.

Experiment 1—A significant difference was not found between mean lesion scores for calves in groups 1 and 2 (PBSS and medium controls; Table 1). Mean lesion scores for calves in group 3 (1×10^9 CFU/ml, recently harvested) and group 4 (10^8 CFU/ml, lyophilized) were significantly ($P < 0.001$) less than the mean lesion scores for calves in groups 1, 2, and 5 (1×10^6 CFU/ml, lyophilized).

Vaccination with live *P haemolytica* (groups 3, 4, and 5) resulted in a significant ($P < 0.01$) increase in antibody responses to somatic antigens (FIAX) and to the CPS (ELISA). Mean LN titers, A_{490} values, and FIAX titers were significantly ($P < 0.01$) higher on day 21 for calves in groups 3, 4, and 5 than for groups 1 and 2. A significant ($P < 0.02$) correlation was found between lesion scores and antibody responses, determined by use of all 3 techniques (r −0.534 to −0.571).

Experiment 2—All group 6 (PBSS control) calves died within 24 hours after challenge exposure, due to severe pneumonia and *P haemolytica* septicemia. One group 8 calf and 1 group 10 calf also died within 72 hours after challenge exposure. Each vaccination dosage enhanced resistance of calves against challenge exposure. Mean lesion scores for calves in groups 7 (5×10^9 CFU, recently harvested), 8 (5×10^8 CFU, lyophilized), 9 (2×10^8 CFU, lyophilized), and 10 (1×10^8 CFU, lyophilized) were significantly ($P < 0.05$) lower than for calves in group 6. Mean LN titers were significantly ($P < 0.05$) higher for calves in groups 7, 8, and 9 than for calves in groups 6 and 10. A significant ($P < 0.01$) correlation was found between lesion scores and antibody titers, as determined by use of all 3 assays (r = −0.566 to −0.709).

Experiment 3—Two injections (days 0 and 7, group 13) of CFU of lyophilized vaccine (2×10^8 CFU) resulted in a lower mean lesion score than did 1 injection (day 0, group 14; Table 1); however, this difference was not significant. The mean lesion scores for calves in groups 13 and 14 were significantly lower ($P < 0.01$) than were scores for calves in group 11 (PBSS control).

Mean LN titers were significantly ($P < 0.05$) greater for calves in groups 13 and 14 than for calves in group 11. Mean antibody responses (FIAX and ELISA) were significantly ($P < 0.05$) greater for calves in group 13 than for calves in groups 11 and 14. High antibody titers (as determined by all 3 assays) significantly ($P < 0.05$) correlated with low lesion scores (r = −0.509 to −0.624).

Discussion

Results of the present study indicate that calves vaccinated with previously lyophilized and reconstituted live *P haemolytica* were protected against transthoracic challenge exposure with *P haemolytica*. A dosage of 1×10^6 to 5×10^8 CFU of lyophilized organisms given subcutaneously, twice at a 7-day interval, enhanced resistance similar to 5×10^9 CFU of recently harvested *P haemolytica*, as seen in the present study and in previous studies.[4,9,11-13] Two vaccinations (5×10^6 CFU) did not enhance resistance. Vaccination of calves once with 2×10^8 CFU of *P haemolytica* elicited a protective immunity similar to that elicited by 2 doses. Generally, the antibody response (FIAX and ELISA) was not as marked in calves given 1 dose as in calves given 2 doses. However, LN titers were comparable for both groups. The mean LN titer on day 0 was slightly higher in calves given 1 dose than in calves given 2 doses; therefore, the higher mean response in calves given 1 dose may have been due to an anamnestic response in several calves.

TABLE 1—Lesion scores and antibody response of calves vaccinated with phosphate-buffered saline solution (PBSS), polypeptone lyophilization medium (PLM), or live or lyophilized *Pasteurella haemolytica* vaccine and challenge exposed with *P haemolytica*

Experiment No.	Group No.	Vaccine	No. of calves	Lesion score (mean* ± SD)	Antibody response (mean ± SD): Quantitative fluorometric immunoassay (somatic antigen)† Day 0§	Quantitative fluorometric immunoassay (somatic antigen)† Day 21	ELISA (carbohydrate-protein subunit)‡ Day 0	ELISA (carbohydrate-protein subunit)‡ Day 21	Leukotoxin neutralization† Day 0	Leukotoxin neutralization† Day 21
1¶	1	PBSS	4	12.3 ± 4.4	0.7 ± 1.9	16.1 ± 1.8	0.18 ± 0.02	0.19 ± 0.02	ND	4.8 ± 1.8
	2	PLM	4	14.0 ± 3.7	0.9 ± 1.4	11.7 ± 1.9	0.18 ± 0.02	0.27 ± 0.07	ND	5.4 ± 2.1
	All Controls (groups 1 and 2)		8	13.1 ± 4.2	0.8 ± 0.5	13.9 ± 1.7	0.18 ± 0.02	0.23 ± 0.06	ND	5.4 ± 2.1
	3	Live¶	4	3.5 ± 1.5	0.3 ± 2.1	146.2 ± 1.6	0.35 ± 0.04	0.77 ± 0.15	ND	45.3 ± 1.8
	4	Lyophilized (5ml)**	6	3.6 ± 1.7	0.7 ± 2.5	163.3 ± 1.3	0.22 ± 0.03	0.63 ± 0.02	ND	71.8 ± 1.9
	5	Lyophilized (5ml)††	6	11.7 ± 7.3	0.7 ± 4.2	163.9 ± 1.1	0.23 ± 0.03	0.58 ± 0.07	ND	35.9 ± 1.3
2‡‡	6	PBSS	5	20.0 ± 0.0	18.0 ± 2.1	23.8 ± 1.5	0.25 ± 0.06	0.32 ± 0.10	ND	4.0 ± 2.7
	7	Live¶	3	5.0 ± 3.0	35.5 ± 1.9	152.9 ± 1.4	0.28 ± 0.13	0.68 ± 0.30	ND	79.4 ± 1.4
	8	Lyophilized (5ml)**	5	9.3 ± 6.6	6.5 ± 4.5	121.6 ± 1.4	0.23 ± 0.04	0.63 ± 0.08	ND	41.7 ± 2.8
	9	Lyophilized (2ml)**	5	6.2 ± 4.7	2.5 ± 4.0	119.0 ± 2.0	0.25 ± 0.06	0.67 ± 0.08	ND	20.9 ± 2.0
	10	Lyophilized (1ml)**	5	8.0 ± 6.9	22.8 ± 1.9	137.2 ± 1.9	0.27 ± 0.02	0.71 ± 0.16	ND	18.2 ± 2.8
3§§	11	PBSS	5	14.1 ± 5.1	6.9 ± 2.9	31.2 ± 1.5	0.29 ± 0.02	0.45 ± 0.16	10.0 ± 11.2	6.9 ± 1.7
	12	Live¶	2	4.8 ± 1.8	10.0 ± 1.9	148.3 ± 1.2	0.37 ± 0.22	0.99 ± 0.36	20.0 ± 12.0	64.0 ± 0.0
	13	Lyophilized (2 doses)‖	5	3.0 ± 1.8	3.0 ± 3.6	86.8 ± 1.4	0.24 ± 0.05	0.69 ± 0.14	6.4 ± 5.3	27.5 ± 2.0
	14	Lyophilized (1 dose)‖	5	5.0 ± 3.2	1.6 ± 2.6	56.8 ± 2.3	0.24 ± 0.07	0.53 ± 0.23	10.0 ± 11.2	20.9 ± 2.0

* Arithmetic mean; † Geometric mean titer; ‡ Mean absorbance at 490 nm; § Day of initial vaccination; ‖ Effect of vaccine concentration; ¶ Removed from fresh culture (5 ml; 1×10^9 colony-forming units [CFU]/ml); ** 1×10^8 CFU/ml; †† 1×10^9 CFU/ml; ‡‡ Effect of vaccine volume; §§ Effect of 1 or 2 doses; ‖ Two milliliters/dose (1×10^9 CFU/ml).

ND = not done; ELISA = enzyme-linked immunosorbent assay.

The lyophilization procedure used in the present study protected the organisms during freeze drying, as did skim milk. Other lyophilization media containing only BSA, dextrose, or both did not protect the organism, as determined by marked reduction in CFU after lyophilization. We decided that skim milk would not be a good medium for vaccine production because heifers or cows vaccinated with such a vaccine potentially might develop a milk allergy at the time of lactation.[22] The PLM was used because it provided excellent growth for *P haemolytica* in vitro and therefore, potentially could induce limited in vivo replication at the injection site.[23] As determined by use of the ELISA, neither polypeptone nor yeast hydrolysate was markedly antigenic. The medium-vaccinated (group 2) calves did not develop a delayed-type hypersensitivity response to the polypeptone. Furthermore, a significant immunoglobulin (Ig)G class antibody response ($P > 0.05$) was not detected against polypeptone or yeast hydrolysate. This does not exclude the possibility that an IgE-type hypersensitivity may develop after immunization with a vaccine containing PLM.

Results of the present study corroborate previous findings in calves vaccinated with PBSS and with live *P haemolytica*.[9,11,12] In previous studies[9,11,12] and in the present study, high serum antibody titers against formalin-killed *P haemolytica* and CPS and high LN titers correlated with resistance to transthoracic challenge exposure with *P haemolytica*. However, when data from calves vaccinated with PBSS, bacterin with added adjuvant (aluminum hydroxide), and live *P haemolytica* were analyzed collectively, high antibody to CPS, and high LN neutralizing titers correlated with resistance, whereas high antibody titers against formalin-killed bacteria did not correlate with resistance.[4,11,12] Calves protected against experimental pneumonic pasteurellosis by use of *P haemolytica* bacterins with added oil adjuvants did not develop significant increases in LN antibody, but did have high serum antibody titers against CPS and formalin-killed bacteria.[24] Therefore, LN antibody, although potentially protective against pneumonic pasteurellosis, may not be the only mechanism of immune-mediated resistance, because antibodies against protein or carbohydrate antigens within the CPS also may be protective.

Resistance against bovine pneumonic pasteurellosis has been enhanced by use of several different lyophilized live *P haemolytica* vaccines.[3,7,8,10] A modified-live *P haemolytica* vaccine (4.5×10^8 CFU) given once intradermally in beef and dairy calves[10,25] induced a significant increase ($P < 0.05$) in mean serum antibody titer against *P haemolytica* somatic antigens 14 days after vaccination; however, 30.4% of vaccinated calves did not develop a detectable serum antibody response to formalin-killed bacteria.[25] Kucera et al[8] found that IM injection of 3×10^9 CFU of a chemically altered organism protected calves against challenge exposure with a bovine herpesvirus-1 and *P haemolytica*, whereas lower vaccine doses induced less resistance against challenge exposure. However, antibody response against *P haemolytica* after vaccination was not determined in that study. Catt et al[3] found that 2 IM injections (at 14-day intervals) of 4×10^8 CFU of a streptomycin-dependent *P haemolytica* mutant in combination with 1×10^6 CFU of mutant *P multocida* induced enhanced resistance to challenge exposure with bovine herpesvirus-1, *P haemolytica*, and *P multocida* and detected a significant increase in serum antibody titer against *P haemolytica* by use of indirect bacterial agglutination.

Wilkie[26] has postulated that high LN antibodies provide protection against pneumonic pasteurellosis. The ability of chemically altered or streptomycin-dependent strains of *P haemolytica* to produce leukotoxin is not known. Streptomycin-dependent bacteria may grow only to a limited extent in vivo because of only a small amount of residual streptomycin in the vaccine.[3,8] Because leukotoxin is the product of actively growing *P haemolytica*, the streptomycin-dependent mutant vaccine may not induce LN antibody titers.[27] Therefore, more studies are needed in which calves are vaccinated with purified antigens to determine the true importance of antibody responses to leukotoxin or specific structural antigens.

Lyophilized cultures of *P haemolytica* can effectively enhance resistance of cattle to transthoracic challenge exposure with the organism. The lyophilization procedure is critical for provision of adequate numbers of viable organisms for immunization. The protective dose of the vaccine given subcutaneously seems to be 1×10^8 to 5×10^9 CFU. As with most vaccines, 2 immunizations seem to be better for inducing antibody production than 1 immunization; however, protection of calves against *P haemolytica* was not significantly better after 2 doses than after 1 dose of vaccine.

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Comparative evaluation of antibodies induced by commercial *Pasteurella haemolytica* vaccines using solid phase immunoassays

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Abstract

The objective of this study was to evaluate the ability of four commercial vaccines to elicit antibodies against the leukotoxin (Lkt), capsular polysaccharide (CP), iron regulated outer membrane proteins (IROMPs), and whole cell (WC) antigens of *Pasteurella haemolytica* A1. Modified double antibody sandwich enzyme linked immunosorbent assays (ELISAs) were developed to measure antibody levels against Lkt, CP and IROMPs. An indirect ELISA was developed to measure the levels of antibody against WC antigens. The ideal cut off points for ELISAs were determined on receiver operating characteristic curves, using sera from 30 calves injected subcutaneously with a live *P. haemolytica* 12296 strain as positive control and sera from 30 colostrum-deprived calves as negative control. The vaccines evaluated were: 'One Shot' (SmithKline Beecham, West Chester, PA) a bacterin-toxoid, 'Presponse' (Langford Laboratories, Guelph, Ontario) a Lkt-rich culture supernatant, 'Once PMH' (BioCor Inc., Omaha, NE) a modified live vaccine, and 'Septimune' (Fort Dodge laboratories, Fort Dodge, IA) an outer membrane extract.

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Thirty, 4–6 week old Holstein calves were randomized into 5 groups to receive one of the four vaccines or a placebo (sterile phosphate buffered saline). The calves were vaccinated intramuscularly on day 0 and on day 14, and bled on days 0, 14, and 28 to measure antibody levels against Lkt, CP, IROMPs, and WC antigens of *P. haemolytica* A1. 'One Shot', and 'Once PMH' vaccinates showed a significant ($P < 0.05$) increase in antibody levels against Lkt at 28 days. 'Once PMH' vaccinates also showed significant ($P < 0.05$) increase in antibody levels against IROMPs at 28 days compared to the other four groups but this increase was not significant over time within the 'Once PMH' group. 'Presponse', 'Once PMH', and 'One Shot' vaccinates showed a significant ($P < 0.05$) increase in antibody levels against CP over time. These groups also had significantly higher antibody levels against CP, compared to controls and 'Septimune' vaccinates at 14 and 28 days ($P < 0.05$).

Keywords: *Pasteurella haemolytica*; Vaccines; ELISA; Receiver operating characteristic curves

1. Introduction

Shipping fever pneumonia in cattle is a multifactorial disease caused by stressful management factors in conjunction with viral and bacterial agents. *Pasteurella haemolytica* biotype A serotype 1 (A1) is the primary agent responsible for the clinical disease and pathophysiologic events characterized by acute lobar fibinonecrotizing pneumonia (Thomson, 1981). With the recognition of the involvement of *P. haemolytica* A1 in the disease, attempts have been made to prevent the disease by vaccines against *P. haemolytica*. Several laboratories have studied the efficacy of a variety of vaccine preparations including live bacteria (Chengappa et al., 1989; Confer et al., 1984; Confer et al., 1986), bacterins (Confer et al., 1987), capsular polysaccharide (CP) (Conlon and Shewen, 1993), outer membrane proteins (Confer et al., 1985; Craven et al., 1991), culture supernatant of *P. haemolytica* (Shewen and Wilkie, 1988; Shewen et al., 1988; Conlon et al., 1991), and recombinant leukotoxin (Conlon et al., 1991) with varying results. Some of these preparations have been licensed by the United States Department of Agriculture (USDA) and are available commercially for use in the cattle industry (Compendium of Veterinary Products, 1995).

An increase in the understanding of the mechanism of pathogenesis of *P. haemolytica* A1 and the host immune response indicate that current vaccines against pasteurella can be improved. The importance of several virulence factors as immunogens in eliciting a protective immune response has also been realized. These virulence factors are: leukotoxin (Lkt), capsular polysaccharide (CP), iron-regulated outer membrane proteins (IROMPs), and whole cell (WC) antigens of *P. haemolytica*. Individual studies evaluating efficacy of the above mentioned virulence factors as vaccines, have claimed that a significant correlation exists between high antibody levels to Lkt (Gentry et al., 1985), CP (Chae et al., 1990; McVey et al., 1990), and outer membrane proteins (Lessley et al., 1985; Craven et al., 1991; Confer et al., 1985), and an overall protection to intratracheal or transthoracic intrapulmonic challenge with live *P. haemolytica*. However, the observations that bacterins are ineffective (Wilkie et al., 1980; Friend et al., 1977) and that an Lkt-rich bacteria-free extract (Presponse) did not show an overall benefit in a controlled field trial (Thorlakson et al., 1990) indicate a need for more studies evaluating the usefulness of the currently available vaccine preparations. Furthermore, the variability in serologic assays for measuring antibody responses to CP, WC antigens, and Lkt among different laboratories has made it difficult to draw logical conclusions on the ability of various vaccine preparations to elicit antibodies against these antigens. A need exists, therefore, for the development and optimization of specific, sensitive, reproducible, and widely accessible assays to measure the levels of antibody against Lkt, CP, IROMPs, and WC antigens induced by a variety of vaccine preparations.

This study was undertaken with the following objectives: (1) To develop and optimize solid phase immunoassays to measure the levels of antibodies against *P. haemolytica* A1 antigens including Lkt, CP, WC antigens, and IROMPs, and (2) to apply these immunoassays to evaluate four commercially available *P. haemolytica* vaccines in terms of their ability to elicit antibody responses against these antigens.

2. Materials and methods

2.1. Enzyme linked immunosorbant assay (ELISA) protocols

All ELISA procedures were optimized in our laboratory following protocols described previously (Colligan et al., 1992) with several modifications.

2.1.1. ELISA to measure antibody levels against CP (anti-CP ELISA)

A modified double antibody sandwich ELISA was used to measure the levels of antibodies against CP of *P. haemolytica* strain 12296 which is biotype A and serotype 1 (A1). The concentrations of reagents used were optimized using a checkerboard ELISA. Briefly, monoclonal antibody (IgM isotype; mAbIIB6) generated in our laboratory (Penaredondo et al., 1988) against the CP of *P. haemolytica* 12296 was used to coat 96-well flat bottom microtiter plates (Costar, Cambridge, MA.). The monoclonal antibody was serially diluted two-fold (1:1,000 to 1:16,000) in 0.05 M carbonate-bicarbonate buffer (pH 9.6) and was aliquoted in 50 μl amounts into all wells of five plates which were then incubated overnight at room temperature to achieve effective coating of the plates. Each plate received a single dilution of mAbIIB6. The plates were washed thrice with pyrogen free deionized water and then blocked at 37°C for 30 min with 50 μl of 5% fish gelatin (Sigma chemical Co., St. Louis, MO.) in phosphate buffered saline (PBS) containing 0.05% tween-20. The plates were washed again as described before. Varying concentrations of CP (1.525 μg/ml to 25 μg/ml), purified from *P. haemolytica* by the method of Adlam et al. (1984), were prepared in blocking buffer and aliquoted at 50 μl quantities into each well. The concentrations of CP varied across columns 2–11 of the ELISA plates so that at least two columns had a similar concentration in each plate. The plates were incubated for 1 h at 37°C and subsequently washed thrice as described before. Serum from a calf injected subcutaneously with live *P. haemolytica* 12296 was used at varying dilutions (1:200 – 1:1,600) as a positive control in rows A–D of the ELISA plates, while serum from a colostrum deprived calf

was used at similar dilutions as a negative control in rows E–H of the plates. The diluted sera were used at 50 μl quantities per well and plates were incubated at 37°C for 1 h. After washing the plates five times as described before, 50 μl of horseradish peroxidase conjugated goat anti-bovine IgG (μ) (Kirkegaard Perry Laboratories, Inc., Gaithersburg, MD.) was added at a dilution of 1:2,000 into each well. The plates were incubated for 1 h at 37°C and washed five times as before. Fifty μl per well of 2,2'-azino-di[3-ethyl-benzthiazoline sulfonate(6)] (ABTS; Kirkegaard Perry Laboratories, Inc., Gaithersburg, MD.) was used as a substrate at 37°C for 10 min. The reaction was stopped with 20 μl per well of 2% sodium dodecyl sulfate.

The optimal concentrations of each reagent were determined on plots for varying concentrations of the reagents for positive and negative control sera and on the basis of maximal positive to negative ratios. A maximum positive to negative ratio of 4.59 was achieved at 1:4,000 dilution of mAbIIB6, 3.125 μg/ml of CP, and 1:200 dilution of serum. Therefore, these concentrations of the reagents were used in all subsequent assays for anti-CP ELISA.

The anti-CP ELISA was optimized for specificity and sensitivity using sera from 30 calves immunized with live *P. haemolytica* 12296 and from 30 colostrum-deprived calves. The optimal cut off points at 1:200 dilution of the serum were determined by receiver operating characteristic curves. All sera from vaccinated and control calves were assayed at a single dilution of 1:200, and the optical density at 405 nm was used as a measure of antibody levels (Confer et al., 1985).

2.1.2. ELISA to measure antibody levels against Lkt (anti-Lkt ELISA)

A modified double antibody sandwich ELISA to measure antibody levels against the Lkt of *P. haemolytica* A1 was developed along the same lines as described for anti-CP ELISA. The Lkt-neutralizing monoclonal antibody (mAb601) was kindly provided by S. Srikumaran (Gentry and Srikumaran, 1991). Leukotoxin enriched culture supernatant (CS) for this assay was obtained from logarithmic phase growth of *P. haemolytica* 12296 using the method of Vega et al. (1987). The maximal positive to negative ratio of 4.0 was achieved at 1:2,000 dilution of mAb601, 5 μg/ml of CS and 1:200 dilution of serum. Optimal cut off points for anti-Lkt ELISA were determined on a receiver operating characteristic curve as described for anti-CP ELISA.

2.1.3. ELISA to measure antibody levels against IROMPs (anti-IROMPs ELISA)

A modified double antibody sandwich ELISA to measure antibody levels against IROMPs of *P. haemolytica* A1 was developed similar to the method described for anti-CP ELISA. Monospecific polyclonal antibodies (anti-IROMP) against the 77 kDa IROMP of *P. haemolytica* was generated and characterized in our laboratory (Srinand et al., 1996). Sodium salicylate extract (SSE) of *P. haemolytica* A1 grown under iron-restricted conditions was prepared by the method of Gilmour et al. (1991) for this assay. The maximal positive to negative ratio of 2.6 was achieved at 1:80 dilution of monospecific polyclonal anti-IROMP, 5 μg/ml of SSE and 1:200 dilution of serum. Optimal cut off points for anti-IROMP ELISA were determined on a receiver operating characteristic curve as described for anti-CP ELISA.

2.1.4. ELISA to measure antibodies against whole cell antigens (anti-WC ELISA)

An indirect ELISA was developed to measure antibodies against WC antigens of *P. haemolytica* 12296. The bacteria were grown to mid logarithmic phase in brain heart infusion (BHI; Difco laboratories, Detroit, MI.) broth. The bacteria were harvested by centrifuging the growth in BHI broth at 1,000 *g* for 45 min. The bacterial pellet was resuspended in 5 ml of phospate buffered saline (PBS; pH 7.4) and serially diluted two fold at varying optical densities (OD). The bacterial suspensions had ODs ranging from 0.1 to 1.0. Formalin was added to these bacterial suspensions at a concentration of 0.4%. The formalinized bacteria were stored at 4°C until used. Optimal concentrations of various reagents to be used for anti-WC ELISA were standardized on the basis of a checker board ELISA as described by Colligan et al. (1992). The optimal concentrations of the reagents were: 0.45 OD of bacterial suspension, 1:200 dilution of serum, 1:2,000 dilution of horseradish peroxidase conjugated goat anti-bovine IgG. These results were based on positive to negative ratios at varying dilutions of each reagent. Standardization of WC ELISA for routine screening was performed using receiver operating characteristic as described for anti-CP ELISA.

2.1.5. Lkt neutralization assay

A colorimetric assay utilizing XTT (Sigma chemical Co., St. Louis, MO.) dye and bovine lymphoma 3 (BL-3) cells as target cells was optimized to determine the Lkt neutralization titers in sera of vaccinated and control calves (Vega et al., 1987). Sera were serially diluted in RPMI-1640 containing 0.1 mM $CaCl_2$ and incubated with 30 units of Lkt per well for 10 min at 37°C, in a 5% CO_2-humidified air atmosphere. The BL-3 cells, at a concentration of 2.5×10^6 per ml, were aliquoted into each well and incubated for 45 min in a 5% CO_2-humidified air atmosphere. A solution of XTT dye (1 mg/ml) containing 5 mM of phenazine methosulfate was aliquoted in 50 μl quantities into each well and incubated in an aerobic atmosphere for 3 h. The plates were read at 450 nm and the percent neutralization for each dilution of the sera were calculated using the formula: $\{1 - [(\text{OD of serum-treated cells} - \text{OD of Lkt plus serum-treated cells})/(\text{OD of untreated cell controls} - \text{OD of Lkt-treated cells})]\} \times 100$. The reciprocal of the serum dilution that had 50% neutralization was considered the neutralization titer (Vega et al., 1987).

2.2. Vaccines

Commercially available vaccines used in this trial were a bacterin-toxoid (One Shot; SmithKline Beecham, West Chester, PA.), an Lkt-rich culture supernatant (Presponse; Langford Laboratories, Guelph, Ontario), a modified live *P. haemolytica*/*P. multocida* vaccine (Once PMH; BioCor Inc., Omaha, NE.), and an outer membrane extract of *P. haemolytica* (Septimune PH-K; Fort Dodge laboratories, Fort Dodge, IA.). These vaccines were purchased directly from the manufacturers and stored at 4°C for the duration of the study. The vaccines used were of the same batch so as to avoid batch to batch variations within vaccine groups. The vaccines were administered intramuscularly at doses recommended by the manufacturers.

An indirect ELISA was performed to quantify the amount of antigenic Lkt in these

preparations. Culture Supernatant (CS) from logarithmic growth phase of *P. haemolytica* 12296 with known leukotoxic activity of 2,307 units/mg dry weight was used as a positive control. Varying concentrations (50 μg/ml–0.2 μg/ml) of CS, One Shot, and Once PMH were coated overnight on 96-well flat bottomed plates (Costar, Connecticut, MA.). By contrast, varying dilutions (1:2–1:1,048) of Presponse and Septimune PH-K were used to coat the plates. Monoclonal antibody (mAb601) against Lkt was used as a primary antibody at a dilution of 1:2,000. The plates were incubated at 37°C for 2 h and subsequently washed thrice with pyrogen free distilled water. Horseradish peroxidase conjugated anti-mouse IgG was used as a secondary antibody at a dilution of 1:2,000 and ABTS was used as a substrate in the ELISA system. The optical densities at varying concentrations of CS were plotted to obtain a standard curve. Concentrations of antigenic Lkt were expressed as units per mg or ml, calculated by extrapolation based on the standard curve established with Lkt present in the CS.

Antigenic leukotoxin concentrations for One Shot, Presponse, Once PMH, and Septimune PH-K were ~353 units/mg, ~110 units/ml, 0 units/mg, and 0 units/ml, respectively (Table 1).

2.3. Experimental animals and vaccination schedule

Thirty Holstein calves (4–6 weeks of age) were obtained from the University of Minnesota dairy barn for this study. The calves were weaned at birth and housed in individual hutches located outside the barn for the entire duration of the study. These calves were free from *P. haemolytica* in their upper respiratory tract and had minimal levels of antibody against the suface antigens of *P. haemolytica* as determined by an indirect ELISA. The calves were randomized into five groups of six calves each and were given intramuscular injections of either Presponse, Septimune PH-K, One Shot, or Once PMH vaccines, or sterile phosphate buffered saline (placebo). The calves were vaccinated twice at 14 day intervals, and bled on days 0, 14, and 28 to measure serum antibody levels against Lkt, CP, IROMPs, and WC antigens. The identity of the vaccine groups was not disclosed to the operator assaying the sera.

2.4. Statistical analysis

Receiver operating characteristic curves (plots of false-positive rates against cut off points) for each ELISA were developed as described by Somoza et al. (1989). Briefly, false positive rates (1-specificity) were plotted against sensitivities at various cut off optical densities starting from negative mean, up to negative mean + 10 standard errors. The ideal cut off point was determined as the point where minimal false positive rate (or maximal specificity) and maximal sensitivity were achieved.

Comparison of mean optical densities from ELISAs, at 1:200 dilution of the sera, between vaccinates and controls at different points in time, was done using least squares means (Fisher's protected least significant differences) method, at 5% rejection level. Similar comparisons over time among vaccinates and controls were performed. Correlation between anti-Lkt ELISA and Lkt neutralization assays were calculated using the Pearson's product moment method using time as a weighting variable. All analyses were performed using the software STATISTICA (Statsoft, Tulsa, OK). In all analyses, a *P*-value of 0.05 or less was considered significant.

3. Results

The receiver operating characteristic curves for ELISAs to measure antibody levels against CP, Lkt, IROMPs, and WC antigens of *P. haemolytica* 12296 are shown in Figs. 1–4, respectively.

3.1. Antibodies against CP

The results of antibody levels against CP of *P. haemolytica* are shown in Table 2. There were no baseline differences among vaccinates or between vaccinates and controls. Once PMH, Presponse, and One Shot vaccinates had significantly higher antibody levels against CP compared to controls on day 14 (post-vaccination). The One Shot vaccinates had significantly higher antibody levels against CP compared to Presponse, Once PMH, and Septimune PH-K vaccinates at this time. On day 28

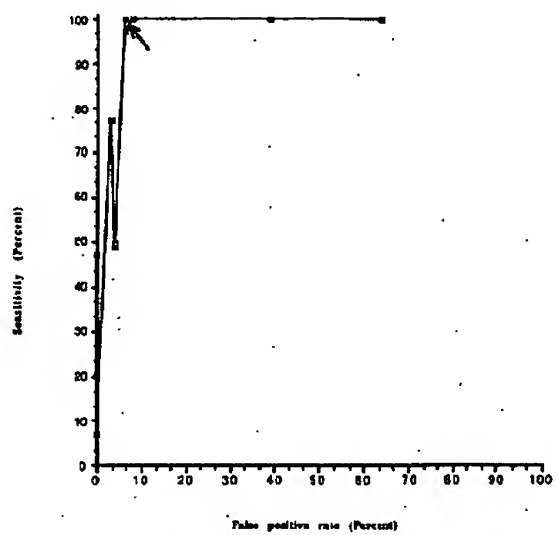

Fig. 1. Receiver operating characteristic curve on anti-CP ELISA. The arrow indicates the cut off point (negative mean + 5 SE) at which minimal false positive rate and maximal sensitivity were achieved.

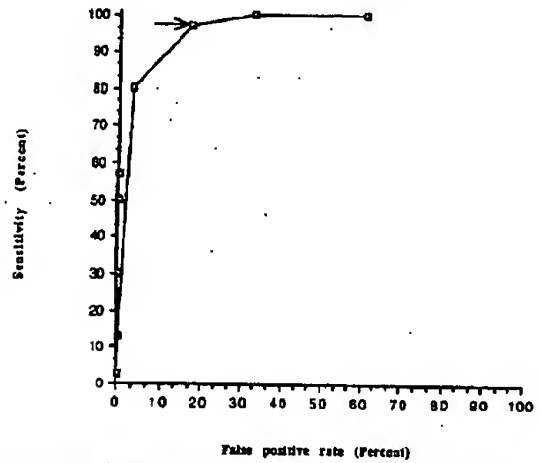

Fig. 2. Receiver operating characteristic curve on anti-Lkt ELISA. The arrow indicates the cut off point (negative mean − 6 SE) at which minimal false positive rate and maximal sensitivity were achieved.

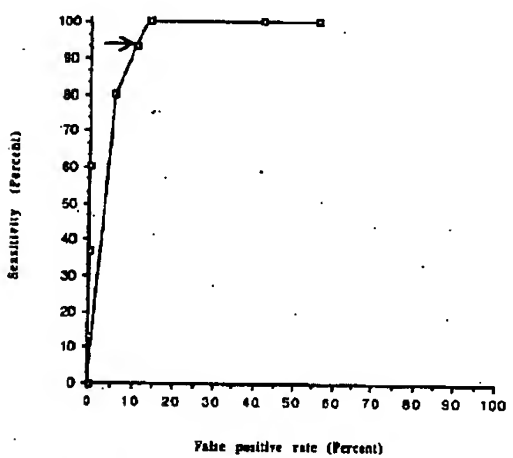

Fig. 3. Receiver operating characteristic curve on anti-IROMP ELISA. The arrow indicates the cut off point (negative mean + 4 SE) at which minimal false positive rate and maximal sensitivity were achieved.

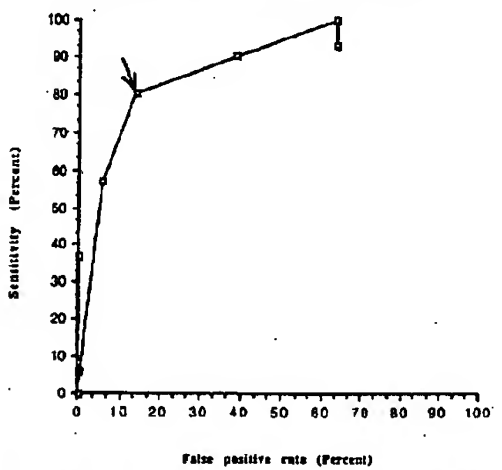

Fig. 4. Receiver operating characteristic curve on anti-WC ELISA.The arrow indicates the cut off point (negative mean + 4 SE) at which minimal false positive rate and maximal sensitivity were achieved.

(post-booster). Once PMH vaccinates had the highest antibody levels against CP followed by Presponse, One Shot, Septimune PH-K vaccinates and controls. There was a significant increase in the antibody levels in animals vaccinated with Once PMH, One Shot, and Presponse compared to Septimune PH-K or controls.

3.2. Antibodies against Lkt

The results of antibody levels against Lkt of *P. haemolytica* by ELISA and neutralization assays are shown in Table 3 and Table 6 respectively. In the ELISA, there were significant increases in the antibody levels against Lkt at day 28 among Once PMH and One Shot vaccinates. These increases were significant over time within Once PMH and One Shot vaccinate groups. Lkt neutralization results also reflected a similar trend in that Once PMH and One Shot vaccinates had significantly higher titers at post booster compared to the other three groups. But the increases in Lkt-neutralizing antibody titers were not significant over time within these vaccine groups. The correlation between Lkt-ELISA and Lkt neutralization was 0.5 ($P < 0.001$).

3.3. Antibodies against IROMPs

The results of antibody levels against IROMPs of *P. haemolytica* are shown in Table 4. Only Once PMH vaccinate group had significantly higher antibody levels against

Table 1
List of *P. haemolytica* vaccines available for use against shipping fever

Vaccine	Company
Bacterins	
Pasteurella haemolytica / multocida (Ph-Pm)	
Pneumosya-H	Colorado Serum, Denver, CO
	Sanofi, Overland Park, KS
	Franklin, Fort Dodge, IA
One Shot	SmithKline Beecham, West Chester, PA
Septimune PH-K	Fort Dodge Laboratories, Fort Dodge, IA
Pasurpro	Coopers, Mundelein, IL
Shipguard	Coopers, Mundelein, IL
Cell-free extracts	
Presponse	Langford Laboratories, Guelph, Ontario
Combinations	
IBR/PI-3/Ph-Pm	Sanofi, Overland Park, KS
Bar-4	Anchor, St.Joseph, MO
Bar-Somnus-2P	Anchor, St.Joseph, MO
IBR/PI-3/BVD/Ph-Pm	Sanofi, Overland Park, KS
Clostridium chauvoei-septicum /Ph-Pm	Colorado Serum, Denver, CO
Discovery 4+Ph	Franklin, Fort Dodge, IA
Resvac/Somubac	SmithKline Beecham, West Chester, PA
Triangle 4+Ph-K	Fort Dodge Laboratories, Fort Dodge, IA
Somnugen-2P	Anchor, St.Joseph, MO
Modified live	
Once PMH	BioCor Inc., Omaha, NE

IBR = Infectious bovine rhinotracheitis
PI-3 = Parainfluenza-3
BVD = Bovine virus diarrhea
Ph-Pm = *Pasteurella haemolytica-Pasteurella multocida*

IROMPs at post booster compared to the other groups. But this increase was not significant within the Once PMH group over time. No other differences in antibody levels against IROMPs were noted.

Table 2
Serum anti-CP antibody levels (least squares means of optical density at 405 nm ± SD) in calves vaccinated with various *P. haemolytica* commercial vaccines

Vaccine	Baseline	Post vaccination	Post booster
Control	$0.15 \pm 0.1^{a,\phi}$	$0.14 \pm 0.11^{a,\phi}$	$0.15 \pm 0.09^{a,\phi}$
Once PMH	$0.17 \pm 0.13^{a,\phi}$	$0.34 \pm 0.14^{b,a,*}$	$0.64 \pm 0.27^{b,\$}$
One Shot	$0.07 \pm 0.04^{a,\phi}$	$0.23 \pm 0.15^{b,*}$	$0.40 \pm 0.30^{b,c,\$}$
Presponse	$0.12 \pm 0.09^{a,\phi}$	$0.21 \pm 0.08^{b,c,\phi}$	$0.40 \pm 0.30^{b,c,\$}$
Septimune	$0.06 \pm 0.02^{a,\phi}$	$0.05 \pm 0.01^{a,\phi}$	$0.09 \pm 0.09^{a,\phi}$

Different alphabetical superscripts indicate significant differences ($P \leq 0.05$) between vaccine type, at each point in time.
Different symbol superscripts indicate significant differences ($P \leq 0.05$) within each vaccine type over time.

3.4. Antibodies against WC antigens

The results of antibody levels against WC antigens of *P. haemolytica* are shown in Table 5. There were no baseline differences among the five groups. At day 14

Table 3
Serum anti-Lkt antibody levels (least squares means of optical density at 405 nm ± SD) in calves vaccinated with various *P. haemolytica* commercial vaccines

Vaccine	Baseline	Post vaccination	Post booster
Control	$0.18 \pm 0.06^{a,\phi}$	$0.16 \pm 0.06^{a,\phi}$	$0.14 \pm 0.14^{a,\phi}$
Once PMH	$0.20 \pm 0.07^{a,\phi}$	$0.20 \pm 0.05^{a,\phi}$	$0.35 \pm 0.2^{b,*}$
One Shot	$0.18 \pm 0.09^{a,\phi}$	$0.21 \pm 0.13^{a,\phi}$	$0.40 \pm 0.30^{b,*}$
Presponse	$0.20 \pm 0.10^{a,\phi}$	$0.20 \pm 0.1^{a,\phi}$	$0.20 \pm 0.10^{a,\phi}$
Septimune	$0.14 \pm 0.02^{a,\phi}$	$0.13 \pm 0.01^{a,\phi}$	$0.12 \pm 0.02^{a,\phi}$

Different alphabetical superscripts indicate significant differences ($P \leq 0.05$) between vaccine type, at each point in time.
Different symbol superscripts indicate significant differences ($P \leq 0.05$) within each vaccine type over time.

Table 4
Serum anti-IROMP antibody levels (least squares means of optical density at 405 nm ± SD) in calves vaccinated with various *P. haemolytica* commercial vaccines

Vaccine	Baseline	Post vaccination	Post booster
Control	$0.15 \pm 0.07^{a,\phi}$	$0.12 \pm 0.08^{a,\phi}$	$0.11 \pm 0.07^{a,\phi}$
Once PMH	$0.15 \pm 0.09^{a,\phi}$	$0.17 \pm 0.03^{a,\phi}$	$0.20 \pm 0.05^{a,\phi}$
One Shot	$0.10 \pm 0.07^{a,\phi}$	$0.10 \pm 0.07^{a,\phi}$	$0.13 \pm 0.08^{a,\phi}$
Presponse	$0.13 \pm 0.05^{a,\phi}$	$0.10 \pm 0.03^{a,\phi}$	$0.12 \pm 0.06^{a,\phi}$
Septimune	$0.1 \pm 0.05^{a,\phi}$	$0.08 \pm 0.02^{a,\phi}$	$0.05 \pm 0.01^{a,\phi}$

Different alphabetical superscripts indicate significant differences ($P \leq 0.05$) between vaccine types, at each point in time.
Different symbol superscripts indicate significant differences ($P \leq 0.05$) within each vaccine types over time.

Table 5
Serum anti-WC antibody levels (least squares means of optical density at 405 nm ± SD) in calves vaccinated with various *P. haemolytica* commercial vaccines

Vaccine	Baseline	Post vaccination	Post booster
Control	$0.10 \pm 0.09^{a,\phi}$	$0.10 \pm 0.10^{a,\phi}$	$0.08 \pm 0.08^{a,\phi}$
Once PMH	$0.13 \pm 0.10^{a,\phi}$	$0.29 \pm 0.24^{b,*}$	$0.30 \pm 0.22^{b,*}$
One Shot	$0.05 \pm 0.04^{a,\phi}$	$0.10 \pm 0.04^{a,\phi,*}$	$0.17 \pm 0.03^{b,*}$
Presponse	$0.10 \pm 0.09^{a,\phi}$	$0.09 \pm 0.08^{a,\phi}$	$0.16 \pm 0.05^{a,\phi}$
Septimune	$0.05 \pm 0.02^{a,\phi}$	$0.04 \pm 0.01^{a,\phi}$	$0.03 \pm 0.01^{a,\phi}$

Different alphabetical superscripts indicate significant differences ($P \leq 0.05$) between vaccine type, at each point in time.
Different symbol superscripts indicate significant differences ($P \leq 0.05$) within each vaccine type over time.

Table 6
Serum Lkt neutralization titers (reciprocal log 2 dilution at 50% neutralization ± SD) in calves vaccinated with various *P. haemolytica* commercial vaccines

Vaccine	Baseline	Post vaccination	Post booster
Control	$3.8 \pm 3.0^{a,\beta}$	$3.8 \pm 3.0^{a,\beta}$	$1.7 \pm 1.3^{a,\beta}$
Once PMH	$2.2 \pm 2.6^{a,\beta}$	$2.4 \pm 2.0^{a,\beta}$	$4.1 \pm 1.7^{b,\alpha}$
One Shot	$1.3 \pm 1.0^{a,\beta}$	$2.9 \pm 0.8^{a,\beta}$	$3.3 \pm 1.5^{b,\alpha}$
Presponse	$2.6 \pm 1.8^{a,\beta}$	$2.4 \pm 0.9^{a,\beta}$	$3.2 \pm 2.2^{a,\beta}$
Septimune	$2.0 \pm 0.4^{a,\beta}$	$1.6 \pm 0.6^{a,\beta}$	$1.7 \pm 0.7^{a,\beta}$

Different alphabetical superscripts indicate significant differences ($P \leq 0.05$) between vaccine type, at each point in time.
Different symbol superscripts indicate significant differences ($P \leq 0.05$) within each vaccine type over time.

post-vaccination, there was a significant ($P < 0.05$) increase in antibody levels among the Once PMH vaccinates compared to controls. At day 28 (post-booster), Once PMH and One Shot vaccinates had significantly higher levels of antibodies against WC compared to controls, Presponse, and Septimune PH-K vaccinates. Temporal trends indicated significant increases in antibody levels over time for Once PMH and One Shot vaccinates.

4. Discussion

Various types of *P. haemolytica* vaccines have been marketed in North America over the past few years. In this study, we evaluated ability of four vaccines to elicit antibody responses against various antigens of *P. haemolytica* A1 including Lkt, CP, IROMPs, and WC antigens using ELISA methodology.

Commercial bacterins have been evaluated in many laboratories in experimental-challenge models with mixed results. While some studies have shown a marked protection (Confer and Panciera, 1994), other studies have demonstrated that bacterins enhanced clinical disease and lesions when vaccinates were challenged with *P. haemolytica* intratracheally (Wilkie et al., 1980). Some studies have demonstrated the benefit of bacterins with oil adjuvants (Confer et al., 1987). A Lkt-rich culture supernatant from log phase growth of *P. haemolytica* (sold as Presponse), on the other hand, has shown excellent protective immunity under laboratory conditions (Shewen et al., 1988, and Shewen and Wilkie, 1988). Additionally in another laboratory study, the same preparation when used with recombinant Lkt, showed better protective immunity than when either preparation was used alone (Conlon et al., 1991). However, a recent field trial (Thorlakson et al., 1990) did not show an overall benefit of using Presponse in calves upon arrival in the feedlots.

The evidence that resistance to pneumonic pasteurellosis correlates with high serum antibody levels against various antigens of *P. haemolytica* e.g., Lkt (Gentry et al., 1985), Lkt and whole cell antigens (Shewen and Wilkie, 1983), CP (Confer et al., 1989), and outer membrane proteins (Confer et al., 1985), stimulated our interest to perform the

present study. Previous studies have utilized ELISAs in some cases and fluorimetric antibody assays in others, with unspecified specificities and sensitivities. This makes it very difficult to compare various studies with respect to the correlation between antibody responses against a variety of *P. haemolytica* antigens and protective immunity. In the present investigation, we report an objective method to standardize ELISAs using receiver operating characteristic curves. This method utilizes the comparison of false positive rates with sensitivities over a range of cut off points so that the operating characteristics (specificity and sensitivity) of an assay at a given cut off point can be determined and used objectively to compare antibody levels at that point.

One Shot, Once PMH, and Presponse vaccine preparations induced significant increases in antibody levels against WC antigens and CP of *P. haemolytica*. This is probably because of the high antigenic content in individual preparations. Other studies using fluorimetric indirect agglutination test have shown similar antibody responses to a variety of preparations of *P. haemolytica*. The finding that animals responded to CP is in agreement with previous reports (Conlon et al., 1993; McVey et al., 1990). There were no differences between vaccinates and controls in terms of their ability to elicit antibody responses against IROMPs. Such a response was not expected from bacterins or from cell free extracts of *P. haemolytica* which were not derived from cultures grown under iron-restricted conditions. Although Once PMH vaccinates had significantly higher levels of antibodies against IROMPs at day 28, this increase was not significant over time within this group. An antibody response to IROMPs was expected in the modified live vaccine group (Once PMH) because of its ability to multiply in the host for at least a few generations at the vaccination site. The modified live vaccine (Once PMH) may not be multiplying at the vaccination site to elaborate adequate antigenic mass or the modified live organisms may not be expressing IROMPs. The observation that only Once PMH and One Shot induced an increase in the antibody levels against Lkt is not surprising because the former is a modified live vaccine while the latter has a higher antigenic (but not bioactive) Lkt content compared to the other two vaccines. However, the highest response against Lkt was seen only at 14 days after booster for both the vaccines. Unfortunately, the manufacturers of both products do not recommend a booster dose, claiming that a boostering effect occurs in the field due to a previous natural exposure of animals to *P. haemolytica*. This hypothesis needs testing in a randomized controlled field trial. The reason for the lack of antibody response to Lkt in the Presponse and Septimune vaccinates may be due to: (a) the lack of adequate amounts of antigenic Lkt in the vaccines, or (b) the presence of other immunodominant antigens like CP in excess in the vaccines which may have diverted antibody responses from Lkt.

Although the claims from individual manufacturers have been made that the vaccines induce high Lkt neutralization titers (in vaccinates), variability in the assays between laboratories in terms of their stringency in the calculation of 50% neutralization, the dye used (trypan blue, MTT, or XTT) or the target cell types and quality may influence the results. In this study, we used the XTT dye assay with BL-3 cells as target cells and were objective in the calculations of 50% neutralization point. All samples were blinded to the operator and assayed in triplicate, which made the test results highly conservative and reliable.

This study did not attempt an experimental-challenge with live *P. haemolytica* to evaluate protective immunity induced by each vaccine type measured by clinical scores as well as reduction in pneumonic lesion scores. In other related studies done in our laboratory with *P. haemolytica*-derived experimental subunit vaccines (Srinand et al., 1996), we found a significant correlation between high antibody levels against Lkt, CP, and IROMPs and a reduction in pneumonic lesion score. Also, the results indicated a significant correlation between high serum and lung antibody levels against Lkt, CP, and IROMPs suggesting that serum antibodies can be used as an indirect measure of local immunity in the lung. Results from other studies demonstrating a correlation between high antibody titers against virulence factors, specifically Lkt and other surface antigens (Shewen and Wilkie, 1988) and reduced pneumonic lesion scores, strongly suggest that these vaccines (One Shot, Once PMH, Presponse, and Septimune) may not be efficacious, especially, when administered at a single dose.

Studies to evaluate these commercial vaccines in a vaccination-challenge model and to correlate the antibody levels against various antigens of *P. haemolytica* A1 with protective immunity are underway in our laboratory. Results from these studies should give definitive answers on the protective efficacy of these commercial vaccines.

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Immunogens of Pasteurella

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ABSTRACT

The family Pasteurellaceae Pohl contains Gram-negative, facultatively anaerobic and fermentative bacteria of the genera *Pasteurella*, *Haemophilus*, and *Actinobacillus*. Approximately 20 different species of the genus *Pasteurella* have been identified using phenotypic and genetic analyses. Of these species, *P. multocida* and *P. haemolytica* are the most prominent pathogens in domestic animals causing severe diseases and major economic losses in the cattle, swine, sheep, and poultry industries. Mechanisms of immunity to these bacteria have been difficult to determine, and efficacious vaccines have been a challenge to develop and evaluate.

Pasteurella multocida of serogroups A and D are mainly responsible for disease in North American poultry and pigs and to a lesser extent in cattle. Fowl cholera in chickens and turkeys is caused by various serotypes of *P. multocida* serogroup A and characterized by acute septicemia and fibrinous pneumonia or chronic fibrinopurulent inflammation of various tissues. Current biologicals in use are live *P. multocida* vaccines and bacterins. Potency tests for avian *P. multocida* biologicals are a bacterial colony count for vaccines and vaccination and challenge of birds for bacterins. Somatic antigens, particularly lipopolysaccharide (LPS), appear to be of major importance in immunity. In North American cattle, *P. multocida* serogroup A is associated mainly with bronchopneumonia (enzootic pneumonia) in young calves; however, it is occasionally isolated from fibrinous pleuropneumonia of feedlot cattle (shipping fever). Biologicals currently available are modified-live vaccines and bacterins. The potency test for vaccines is bacterial colony counts. The test for bacterin potency is vaccination and challenge of mice. Important immunogens have not been well characterized for *P. multocida* infection in cattle. In swine, *P. multocida* infection is sometimes associated with pneumonia; however, its major importance is in atrophic rhinitis. A protein toxin (dermonecrotic toxin), produced by toxigenic strains of *P. multocida* types A and D, and concurrent infection with *Bordetella bronchiseptica* appear to be the major factors in development of atrophic rhinitis. Currently available biologicals are bacterins and inactivated toxins (toxoids). The toxin appears to be the major immunogen for preventing atrophic rhinitis. There are, however, no standardized requirements for potency testing of *P. multocida* type D toxoid.

Various serotypes of *P. haemolytica* biotype A are responsible for severe fibrinous pleuropneumonia of cattle and sheep, occasionally septicemia of lambs, and mastitis in ewes. Several serotypes of *P. haemolytica* biotype T are isolated from acute septicemia of lambs. The currently available *P. haemolytica* biologicals are modified-live vaccines, bacterins, bacterial surface extracts, and culture supernates that contain an exotoxin (leukotoxin). Most biologicals contain *P. haemolytica* biotype A serotype 1; however, biotype T serotypes 3 and 4 are occasionally included. As with *P. multocida* vaccines, the potency test for a *P. haemolytica* vaccine is a bacterial colony count. There are no stan-

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dard guidelines for potency tests for *P. haemolytica* bacterins or extracts and supernate biologicals. The major immunogens for *P. haemolytica* appear to be the leukotoxin, capsule, outer membrane proteins, and iron-regulated proteins.

INTRODUCTION

The family *Pasteurellaceae* Pohl contains Gram-negative, facultatively anaerobic and fermentative bacteria of the genera *Haemophilus, Actinobacillus* and *Pasteurella*. Members of the genus *Pasteurella* cause septicemia, respiratory disease, and mastitis in numerous domestic animal species (Confer et al., 1990). *Pasteurella* spp. have been associated with disease and attempts to develop efficacious biologicals for this group of bacteria date back to the time of Pasteur; however, development of such vaccines has been a formidable task.

A complete description of the taxonomy of the genus *Pasteurella* is beyond the scope of this review (Mutters et al., 1989). However, a brief review of several key points is warranted. As described in *Bergey's Manual of Systematic Bacteriology* (Carter, 1984), the genus *Pasteurella* can be divided among six species based on beta hemolysis, growth on MacConkey's agar, indole production, urease activity, gas from carbohydrates, and acid production from lactose or mannitol. These species are *P. multocida, P. haemolytica, P. pneumotropica, P. ureae, P. aerogenes,* and *P. gallinarum*. Phenotypic properties, however, only account for expression of 10–20% of the genome, and molecular techniques offer more critical analysis of relatedness among species. Therefore, the *Pasteurellae* have been examined using such techniques as DNA/DNA hybridization (Mutters et al., 1986), 2-dimensional protein electrophoresis (Olsen et al., 1987), and rRNA analysis (Dewhirst et al., 1992). Such analyses have resulted in reclassification of several *Pasteurella* species into 11 species in the genus *Pasteurella sensu stricto,* which can be differentiated using certain common phenotypic properties beyond those used in Bergey's manual (Mutter et al., 1989). One of the major organisms affected in this classification is *P. multocida*, which is no longer classified merely as a single bacterial species isolated from a wide variety of diseases in several animal species. Appropriate subspecies of *P. multocida* are now recognized, and these tend to be relatively animal species specific. Several traditional *Pasteurella* species, including *P. haemolytica*, were excluded from *P. sensu stricto,* and their classifications as *Pasteurella* remain questionable especially in light of recent rRNA analyses (Dewhirst et al., 1992). In this review, I will concentrate on immunogens of *P. multocida* and *P. haemolytica* (as traditionally classified) as they relate to development of efficacious biologicals for control of domestic livestock in North America.

Pasteurella multocida

Antigenic typing

P. multocida is classified by capsular (serogroup) and somatic (serotype) antigen typing (reviewed in Rimler and Rhoades, 1989). Encapsulated *P. multocida* can be separated into five serogroups (A, B, D, E, and F) by passive hemagglutination of erythrocytes coated with crude cell extracts containing capsule specific antigen which includes lipopolysaccharide (LPS) and nonantigenic polysaccharide (Carter, 1968). Somatic serotyping of *P. multocida* is most commonly done by gel diffusion precipitin tests (Heddleston et al., 1972). This system recognizes sixteen serotypes and uses chicken antisera made against *P. multocida* bacterins reacted against heat-stable antigens from formalinized-saline suspended bacteria. Specific somatic antisera can be obtained from chickens immunized with purified LPS (Rimler, 1984).

Immunogens

Purified *P. multocida* capsule behaves as a hapten when injected in most animals and has not been extensively investigated as an immunogen against diseases in North America livestock. *P. multocida* LPSs have similar chemical and biological properties to the R-type LPSs of other gram negative bacteria (Lugtenberg et al., 1984). Purified *P. multocida* LPS is antigenic; however, the extent of antibody response following immunization depends on animal species inoculated, LPS type used, and route and method of inoculation (Rimler and Phillips, 1986). In addition, protection afforded by immunization with *P. multocida* LPS is somewhat animal species dependent. In general, *P. multocida* LPS seems to be a major immunogen in birds (Rhoades and Rimler, 1989). However, Tsuji and Matsumoto (1988) suggested that a LPS-protein complex is essential for induction of immunity against *P. multocida* infection in turkeys. The role of LPS as an immunogen in mammals remains controversial. Mice, cattle, and rabbits have not been readily protected against *P. multocida* infection following immunization with LPS (Rimler and Rhoades, 1989). Recently, Wijewardana et al. (1990) found that a bactericidal monoclonal antibody (mAb) against LPS completely protected mice against homologous challenge with live *P. multocida*. However, monoclonal antibodies to LPS that were opsonic but not bactericidal only partial protected mice against *P. multocida* challenge (Ramdani and Adler, 1991). Lu et al. (1991 b) failed to protect mice against *P. multocida* infection by passive transfer of affinity-purified rabbit anti-LPS serum.

Recently, outer membrane proteins (OMPs) of *P. multocida* have been studied as potential immunogens. The immunogenic role of these proteins has been suspected for years; however, LPS and capsular contamination of OMPs have limited interpretation of their immunogenic potential. Lugtenberg et al. (1984) demonstrated three envelope protein profiles of *P. multo-*

cida isolates from swine. Profiles differed primarily on the migration of a surface protein (protein H) with molecular mass of approximately 36–38 kDa (Lugtenberg et al., 1986). Knights et al. (1990) isolated outer membranes of the various serogroups of *P. multocida* and demonstrated that their electrophoretic patterns were markedly different from those of *P. haemolytica*. Iron-regulated OMPs were demonstrated in *P. multocida* and appear to be siderophore receptors (Ikeda and Hirsh, 1988; Choi-Kim et al., 1991). Production of those proteins in vivo was suggested by their demonstration with convalescent sera from *P. multocida*-infected turkeys.

The immunogenic role of *P. multocida* OMPs has been best characterized in rabbits. Lu et al. (1988) demonstrated that rabbits mounted major antibody responses against 5 *P. multocida* OMPs (27, 37.5, 49.5, 58.7, and 64.4 kDa). They further demonstrated that vaccination with *P. multocida* outer membranes protected rabbits against homologous challenge (Lu et al., 1991 c), and protection seemed to be due to antibodies against OMPs and not LPS (Lu et al., 1991 b). More specifically, a mAb against a 37.5 kDa OMP protected both mice and rabbits against *P. multocida* challenge (Lu et al., 1991a). Additionally, Truscott and Hirsh (1988) demonstrated a 50 kDa *P. multocida* OMP that was antiphagocytic. Turkeys given antibodies specific for that OMP were protected against challenge. Abdullahi et al. (1990), however, failed to demonstrate in *P. multocida*-challenged mice any correlation between protection and antibody response to OMP from bovine isolates of *P. multocida*. Membrane-associated cross-protection factor(s) have been identified in *P. multocida* harvested from the blood of turkeys dying of experimental fowl cholera (Rimler and Rhoades, 1989b). These factors stimulate heterotypic immunity to *P. multocida*. Finally, the immunogenic role of iron-regulated *P. multocida* OMP has not been investigated.

Certain *P. multocida* isolates of the A and D serogroups produce antigenically similar protein toxins (approximately 145 kDa) that are toxic for bovine embryonic lung and Vero cells in vitro, are lethal for rodents and birds, induce osteolysis of swine turbinate bones, and produce hemorrhage and necrosis when injected into guinea pig skin (Rimler and Brogden, 1986; Rimler and Rhodes, 1989). Because of the latter effect, the toxin was called dermonecrotic toxin; however, the term *P. multocida* toxin (PMT) is in current use. Although PMT has many characteristics of an exotoxin, it is not secreted from living intact *P. multocida* (Dali et al., 1991), but must be extracted from bacteria by sonication.

PMT has been demonstrated in *P. multocida* isolates from various animal species; however, it is mainly a virulence factor in atrophic rhinitis of pigs. As an immunogen, inactivated PMT (toxoid) induces protection against the lethal effects of PMT in rats and mice (Thurston et al., 1991) and against experimental atrophic rhinitis in pigs (Foged et al., 1989). MAbs against PMT can neutralize its lethal effects in mice (Foged, 1988). The gene for PMT has been cloned and expressed in *Escherichia coli* and characterized (Petersen and Foged, 1989). A deletion mutant of PMT, which is deficient in 121 amino acids in the amino-terminal quarter of PMT, had markedly reduced toxicity for cell culture, mice, and guinea pig skin; however, it retained its immunogenicity for mice and gilts (Nielsen et al., 1991; Petersen et al., 1991). In those studies, vaccinated mice were protected against the lethal effects of PMT, and pigs born to vaccinated gilts had enhanced resistance against experimental atrophic rhinitis.

Poultry biologicals

Various somatic types and serogroups of *P. multocida* especially serogroup A, serotypes 1, 3, and 4, are recognized as the primary cause of fowl cholera in chickens and turkeys (Rhoades and Rimler, 1989). Disease may manifest as an acute septicemia characterized by disseminated intravascular coagulation, petechial to ecchymotic hemorrhages, multifocal hepatic and splenic necrosis, and fibrinous pneumonia. Chronic fowl cholera occurs as localized fibrinopurulent exudate and necrosis in a variety of locations including sinuses, air sacs, lungs, wattles, foot pads, and bones and joints. *P. multocida* factors that may be important for virulence are LPS (Rimler et al., 1984), capsule (Tsuji and Matsumoto, 1989), plasmids, and resistance to complement-mediated bacteriolysis (Lee et al., 1991).

Biologicals for immunization of poultry against *P. multocida* are currently of two types: bacterins and attenuated vaccines. Bacterins induce somatic type-specific immunity, whereas vaccines confer some degree of cross-serotypic immunity. *P. multocida* bacterins are potency tested in chickens or turkeys using a two-stage test. Stage one uses twice-vaccinated and unvaccinated birds challenged intramuscularly with an appropriate virulent reference serotype not less than 14 days after the last vaccination. If 8 or more unvaccinated birds die, then the test is valid and results are evaluated as in Table 1. Stage 2 is conducted like stage 1 and evaluated as in Table 1. Potency test for the avian *P. multocida* vaccine is an arithmetic mean count of colony forming units (CFU) of bacteria. Vaccines must contain CFUs greater than in the test vaccine used in an immunogenicity study conducted similar to the bacterin

Table 1
Potency test evaluation for avian *Pasteurella multocida* bacterins

Stage	Number of vaccinates	Cumulative number of vaccinates	Cumulative total number of dead vaccinates for	
			Satisfactory serial	Unsatisfactory
1	20	20	≤6	≥9
2	20	40	≤15	≥16

potency tests. During the expiration period, the final vaccine samples must contain CFUs at least two-times greater than that used to immunize birds in the immunogenicity test.

The major *P. multocida* immunogen in birds appears to be LPS based on the ability to immunize birds against disease using extracted LPS (Rhoades and Rimler, 1989). However, the role of non-LPS antigens in stimulating immunity to avian *P. multocida* isolates is not known. Several attempts have been made to correlate antibody responses to *P. multocida* with resistance to challenge. Avakian et al. (1986,1989) showed positive correlation between antibody responses to capsular and KSCN-extracted *P. multocida* antigens and resistance of broilers to challenge but showed no correlation between protection and antibody titers to sonicated bacteria. Schlink and Olson (1989) demonstrated correlations between high antibody titers in a microagglutination test and survival of infected turkeys. Determination of the immunogenic role of non-LPS such as capsule, OMP, and in vivo expressed antigens (Glisson and Cheng, 1991) such as the membrane-associated cross-protection factor(s) are critical to understanding immunity to fowl cholera.

Cattle biologicals

P. multocida serogroups B and E are associated with hemorrhagic septicemia (Carter and De Alwis, 1989), whereas respiratory disease is mainly associated with serogroup A (Frank, 1989). Hemorrhagic septicemia is not an important disease in the United States and will not be considered further in this review. *P. multocida* serogroup A is most commonly associated with a fibrinous bronchopneumonia that is less fulminating than the fibrinous pleuropneumonia associated with *P. haemolytica* infection (Dungworth, 1985). *P. multocida* can be isolated from bronchopneumonia in feedlot cattle or from enzootic pneumonia of calves less than 6 months old.

Biologicals available for *P. multocida* in cattle are bacterins and vaccines, usually in combination with *P. haemolytica*. The potency test for non-avian *P. multocida* bacterins is vaccination and challenge of mice. An acceptable potency for mice is 1/20 of the least dose recommended for other animals. The test bacterin is tested against a standard bacterin using at least three fivefold dilutions. Each bacterin dilution is tested in 20 mice by 2 intraperitoneal injections 14 days apart. Mice are challenged intraperitoneally 10–12 days after the second vaccination with 100–10 000 LD_{50} of virulent *P. multocida*. The relative potency (RP) of the test bacterin is calculated as follows: RP = reciprocal of 50% endpoint dilution (highest dilution protecting 50% of the mice) of test bacterin/reciprocal of 50% endpoint dilution of the standard. If the RP is <0.50, than the bacterin being tested is unsatisfactory. *P. multocida* vaccines are potency tested by determination of CFUs in the vaccine. Standards are set as described for the avian vaccines. The major difference being that the reference immunogenicity studies are conducted in calves using a respiratory challenge.

Little has been published about *P. multocida* immunogens of importance for protection against bovine respiratory disease. Limited information is available on immunogens of *P. multocida* isolates from hemorrhagic septicemia (Carter and De Alwis, 1989; Dawkins et al., 1991). Capsular antigen (Nagy and Penn, 1976), LPS or LPS-protein complex, and various proteins (Dawkins et al., 1991) have been suggested as important immunogens for *P. multocida* serogroups B and E. Because of the difficulty in protecting mice with *P. multocida* LPS and the similarity between *P. multocida*-induced respiratory disease in cattle and that seen in the rabbit, OMP (Lu et al., 1991a, b, c) should be investigated as potential immunogens for cattle.

Swine biologicals

P. multocida serogroups A and D are associated with fibrinous bronchopneumonia, lymph node abscesses, and atrophic rhinitis in swine (Dungworth, 1985). The major economically important disease is atrophic rhinitis, a disease of young swine characterized by bacterial-induced atrophy of nasal turbinates (Chanter and Rutter, 1989). Turbinate and mucosal lesions result in clinical signs of upper respiratory disease and poor weight gain. When lesions are severe, facial distortions such as a twisted snout may be seen. Severe atrophic rhinitis occurs when the nasal cavity is colonized by large numbers of toxigenic *P. multocida*, particularly serogroup D. Colonization is enhanced by concurrent nasal infection with cytotoxin-producing *Bordetella bronchiseptica* (Chanter, 1990). The PMT induces degeneration and necrosis of osteoblasts with subsequent osteoclastic osteolysis of the turbinate bones, predominately the ventral turbinates (Dungworth, 1985). Epithelial hyperplasia accompanies the bony changes. PMT was not toxic for swine alveolar macrophages in vitro (Pifoan, 1986), and the role of PMT in swine pneumonia is unknown.

Biologicals currently available for *P. multocida* in swine are bacterins with and without PMT, often packaged in conjunction with other bacterins such as *B. bronchiseptica*, *Erysipelothrix rhusiopathiae*, and *Actinobacillus pleuropneumoniae*. The efficaciousness of *P. multocida* bacterins without PMT in controlling swine pneumonia or atrophic rhinitis is questionable (Chanter and Rutter, 1989). However, immunity to atrophic rhinitis seems to be readily induced passively with PMT-specific antisera (Chanter and Rutter, 1989) and actively with biologicals containing *P. multocida* PMT toxoids (Foged et al., 1989) or deletion mutants of recombinant PMT (Nielsen et al., 1991; Petersen et al., 1991). Currently there is no approved standard potency test for *P. multocida* toxoid vaccines.

PMT is well established as the major immunogen of *P. multocida* in atrophic rhinitis. It is imperative, therefore, that potency tests for PMT biologicals be

developed. To effectively do this will require agreement on a standard model for atrophic rhinitis, standardization of a method of measurement of PMT antigenic mass in a biological preparation, and determination of the required dose of PMT antigen to protect piglets against challenge. The intranasal acetic acid/*P. multocida* challenge of specific-pathogen-free pigs is widely accepted for vaccination/challenge trials (Chanter and Rutter, 1989). Also, Ackermann et al. (1991) recently described a model of atrophic rhinitis using intranasal inoculation of pigs with a sterile sonicate of *B. bronchiseptica* followed by live, toxigenic *P. multocida*. Immunogenicity studies should be conducted using an accepted model, whereby pigs would be vaccinated with varying doses of PMT toxoid whose specific antigenic mass had been quantified using mAbs to PMT in an antigen capturing assay such as an enzyme-linked immunosorbent assay. The minimal antigenic dose required to protect a predetermined number of pigs could be determined and set as the standard for future biologicals. Potency tests would involve the antigenic quantification of PMT toxoid in biologicals and comparison to the standards determined by the immunogenicity study.

Pasteurella haemolytica

Antigenic typing

P. haemolytica can be typed according to capsular antigens into 16 serotypes using indirect hemagglutination or a rapid plate agglutination test (Frank, 1989). Furthermore, *P. haemolytica* isolates can be biotyped as A or T based on colony morphology and carbohydrate fermentation. Recent studies of OMP and the genome of *P. haemolytica* demonstrated marked differences in electrophoretic patterns and nucleic acid sequences between the A and T biotypes (Dewhirst et al., 1992; Knights et al., 1990). *P. haemolytica* biotype T has been reclassified as *Pasteurella trehalosi*. Only *P. haemolytica* biotype A will be considered in this review.

Immunogens

P. haemolytica has numerous potential immunogens. Those with the most potential for stimulating immunity include capsular polysaccharide (Adlam et al., 1984), LPS (Rimsay et al., 1981), OMPs (Squire et al., 1984; Knights et al., 1990), fimbriae (Morch et al., 1987), iron-regulated proteins (Gilmour et al., 1991), and a secreted leukotoxin ([LKT] Shewen and Wilkie, 1985). Two approaches have been used for determining the importance of various immunogens for stimulation of immunity to *P. haemolytica*. First is the vaccination of cattle, sheep, or goats with purified or relatively purified antigens followed by challenge with virulent *P. haemolytica*. The second approach uses sera from cattle previously vaccinated with various biologicals and challenged. The antibody responses to specific antigens are quantitated

and statistically correlated with the lesion score obtained after challenge. Thus, a significant correlation between a high antibody response and resistance to challenge can be used as a predictor of the importance of an antigen in stimulating immunity.

Antibody responses to *P. haemolytica* fimbriae have not been documented. Capsular polysaccharides from five *P. haemolytica* serotypes were purified and characterized (Adlam et al., 1989). *P. haemolytica* A1 capsular polysaccharide is a virulence factor that interferes with phagocytosis and killing of *P. haemolytica* (Czuprynski et al., 1991) and complement-mediated bacteriolysis (Chae et al., 1990). Immunization of ruminants with *P. haemolytica* capsular polysaccharide or live or killed whole cell preparations results in an antibody response to the capsule. Studies in my laboratory indicated that antibody responses to *P. haemolytica* capsular polysaccharide inconsistantly correlated with resistance to experimental challenge in calves vaccinated with various experimental vaccines (Confer et al., 1989). Recently, Conlon and Shewen (1991a) reported that vaccination of calves with purified capsular polysaccharide was ineffective at protecting calves against *P. haemolytica* challenge.

P. haemolytica LPS has classical endotoxin function in vivo (Confer et al., 1990), and it can alter leukocyte function, and is toxic to bovine endothelial cells in vitro (Confer and Simons, 1986a; Paulsen et al., 1989). Antibody responses to the LPS O-antigen are readily detected in calves vaccinated with live and killed *P. haemolytica* biologicals; however, the intensity of antibody responses to LPS did not correlate with resistance to experimental challenge (Confer et al., 1986b). Antibody responses to the toxic lipid A moiety were not demonstrated.

The antibody responses to *P. haemolytica* OMP have been incompletely characterized. In my laboratory, resistance to experimental challenge was enhanced by vaccination with surface extracts of *P. haemolytica* (Confer et al., 1989), and antibody responses to protein antigens in those extracts correlated with resistance. Mosier et al. (1989) showed that high antibody responses to several proteins in the surface extract correlated with resistance to experimental challenge. The highest correlations were for antibody responses to proteins with molecular masses of 86, 66, 49, and 31 kDa. Several of those proteins have molecular masses equivalent to major OMP of *P. haemolytica* (Knights et al., 1990). Recently, Morton et al. (1990) showed that vaccination of cattle with *P. haemolytica* OMP-enriched preparations induced serotypic immunity against experimental challenge.

Iron-regulated proteins of *P. haemolytica* have been described (Deneer and Potter, 1989; Donachie and Gilmour, 1988; Lainson et al., 1991). The 100 and 70 kDa proteins are located in the outer membrane of *P. haemolytica* grown in vivo or under iron-restricted in vitro conditions. Recently, a 35 kDa iron-regulated periplasmic protein was demonstrated in *P. haemolytica* A2

(Lainson et al., 1991). Gilmour et al. (1991) demonstrated that sodium salicylate extract vaccines of *P. haemolytica* A2 prepared from bacteria grown under iron-restricted conditions protected sheep better than similar vaccines prepared from *P. haemolytica* grown in media containing iron.

The *P. haemolytica* LKT has received much acclaim as an immunogen against pneumonic pasteurellosis of cattle. The LKT is a member of the RTX family of toxins (Lo, 1990), is lytic for ruminant leukocytes (Clinkenbeard et al., 1989), and is, therefore, thought to have a major role in the pathogenesis of *P. haemolytica*-induced pneumonia. The genes for LKT have been cloned and sequenced (Lo et al., 1985). Antibodies to LKT are not *P. haemolytica* serotype specific. Vaccination of cattle with live *P. haemolytica* or culture supernates induced high neutralizing antibody titers to LKT, and high titers correlated with resistance to experimental or natural infection with *P. haemolytica* (reviewed in Confer et al., 1988). Shewen and Wilkie (1988) demonstrated that antibodies to surface antigens as well as to LKT were important in inducing protection in experimental pneumonic pasteurellosis. Conlon and Shewen (1991b) recently demonstrated that the addition of recombinant LKT to a supernate vaccine enhanced protection against experimental challenge. In my laboratory and others, however, protection against experimental challenge was seen in cattle vaccinated with *P. haemolytica* biologicals that did not contain LKT and neutralizing antibodies to LKT were not detected (reviewed in Confer et al., 1988).

P. haemolytica biologicals

Various serotypes of *P. haemolytica* biotype A have been isolated from severe fibrinous pleuropneumonia of cattle and sheep and mastitis and septicemia of sheep (Confer et al., 1990) . For the purpose of this review, I will concentrate on the major disease problem associated with *P. haemolytica* in North America, fibrinous pleuropneumonia or shipping fever of cattle. *P. haemolytica* A1 is the most common serotype isolated from shipping fever. This disease is usually a more fulminating and potentially fatal pneumonia than that produced by *P. multocida* infection. Death losses in feedlot and stocker cattle and economic losses to the industries can be severe. The pathogenesis of the *P. haemolytica*-induced pneumonia usually requires stress and/or concurrent viral infection and details have been previously described (Frank, 1989). Although the pathogenesis has not been completely elucidated, LKT, by lysing resident and incoming leukocytes, probably contributes to necrosis seen in the pulmonary alveoli. Endotoxin probably contributes to vascular damage and systemic signs of illness (Confer et al., 1990).

Currently available *P. haemolytica* biologicals are modified-live vaccines, bacterins, bacterial surface extracts, and culture supernates that contain LKT. Most biologicals in the U.S. contain *P. haemolytica* A1; however, *P. haemolytica* T3 and T4 are occasionally included. Currently, potency testing of live

P. haemolytica vaccines is by determination of CFUs. Live *P. haemolytica* vaccines protect cattle well against experimental pneumonic pasteurellosis, but they can have undesirable side effects such as fever, localized abscesses, and lameness (Confer et al., 1986c). Because of these side effects, many of the newer *P. haemolytica* biologicals use the subunit, extract, or supernate approach. There is no current standard for potency tests for non-living *P. haemolytica* biologicals. Development of an acceptable potency test is important for assuring future efficacy.

Two key issues must be addressed in developing *P. haemolytica* potency tests for non-living biologicals, challenge models and critical immunogens. First is determining acceptable challenge models for immunogenicity studies. Results obtained in mouse models in the past, using intraperitoneal vaccination/challenge, had little or no relevance to respiratory disease of cattle. Numerous respiratory challenge models have been used in calves experimentally, and these have been reviewed (Frank, 1989). Those models vary as to the age of cattle used, route of challenge and method of challenge delivery, whether calves are colostrum deprived or not, and the use of stress or virus exposure prior to challenge. It is my observation that neonatal calves do not respond to *P. haemolytica* vaccination and challenge the same as weanling cattle. Their immune responses are less intense, they are highly susceptible to low challenge doses, and individual animal variation is marked (Confer, unpublished data). Because pneumonic pasteurellosis is mainly a disease of weanling cattle, it would be desirable to conduct immunogenicity studies or potency tests in the older cattle. Use of weanling cattle is not without problems, however, they cost more to use and often have preexisting naturally acquired antibodies to *P. haemolytica* or can develop them prior to vaccination. Studies in my laboratory have been conducted with a transthoracic challenge model. This is an unnatural route of infection but has certain advantages over more conventional routes of challenge including rapidity of the challenge procedure, ease of quantifying lesions, and the ability to readily differentiate experimental from naturally acquired pneumonia. The infectious bovine rhinotracheitis virus/intratracheal or intrabronchial *P. haemolytica* challenge models are commonly used. These models have merit due to their natural route of infection and similarity to the natural disease in terms of clinical signs and lesions. Procedures for disease scoring varies among laboratories. Determination of a standard animal challenge model should help in comparison of data collected by various laboratories.

With the high cost of experimentation in cattle and society's increasing concern for animal welfare, it would be desirable to develop in vitro potency tests for non-living *P. haemolytica* biologicals. This brings me to the second issue. What are the important immunogens for inclusion in a *P. haemolytica* biological? If such a question could be answered with surety, then antigens could be quantified in vitro and compared to known standards determined in

immunogenicity studies. Unfortunately, the question cannot be answered with surety. Data indicate that surface antigens – particularly capsule, OMP, and iron-regulated proteins – and LKT are most likely the important immunogens. Immunity probably requires antibody to combinations of those immunogens. Because OMP stimulate homotypic immunity and capsules of various serotypes appear to be serotype specific, LKT may be of major importance for stimulating cross protection against various serotypes. Therefore, determination of the immunizing doses and relative concentrations of the important immunogens must be a major consideration of future *P. haemolytica* research.

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The nature and detection of mycoplasmal immunogens

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ABSTRACT

Mycoplasmal infections are important causes of disease in cattle, swine, sheep, goats and poultry. Vaccination has been shown experimentally to induce protection against challenge with several mycoplasmas, and vaccines have been used to control naturally occurring mycoplasmal disease in swine, sheep, goats and poultry. Immune responses to mycoplasmal immunogens have been determined using ELISA and immunoblotting as well as other serologic techniques. However, the importance of specific immunogens as virulence factors or putative protective immunogens has generally not been determined. Investigations are underway to determine the pathogenic mechanisms and identify important virulence factors involved in mycoplasmal disease. Examples are discussed of investigations with *Mycoplasma hyopneumoniae* from our own laboratory. We have utilized ATP luminometry in attempts to develop better methods for quantitation of growth of *M. hyopneumoniae* and competitive ELISA as a potential method for *in vitro* quantitation of specific important immunogens.

INTRODUCTION

Each species of livestock and companion animals has a unique flora consisting of several mycoplasmal species. Most of these species occur as inhabitants of the mucosal membrane secretions. Some of the mycoplasmas occurring in livestock are important pathogens causing substantial economic loss, whereas others have little or no importance as causes of disease. The predominant disease manifestations are those of respiratory disease, arthritis, reproductive failure and mastitis. Among the most important mycoplasmal species are *M. gallisepticum* in chickens and/or turkeys; *M. hyopneumoniae* in swine; *M. bovis*, *M. dispar*, and *M. mycoides* subsp. *mycoides* SC in cattle; and, *M. agalactiae* and *M. capricolum*/F38 mycoplasma in sheep and/or goats. Protection has been demonstrated with experimental vaccines, and vaccines are used in the field with most of these important animal mycoplasmoses. How-

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